

# DIETARY FISH AND HAEMOSTASIS

Indications of  
anti-thrombotic properties

Margareta Thorngren



Lund 1983



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## Indications of anti-thrombotic properties

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| <b>Abstract</b><br><p>It has been proposed that <math>\omega</math>-3 fatty acids, mainly eicosapentaenoic acid (EPA) protects the Eskimos against cardiovascular disease by reducing thromboxane <math>A_2</math> (TXA<math>_2</math>) production by platelets and thereby diminishing their aggregability. The purpose of the present study was to elucidate possible effects in man of dietary fish, rich in EPA, on haemostasis. Two novel approaches for evaluating primary haemostasis were developed. The Simplate technique for measuring skin bleeding time was adapted to quantify both the <i>in vivo</i> local TXA<math>_2</math> production during bleeding and the volume of the emerging blood as a function of time. Healthy men were maintained for 6 or 11 weeks on a normal diet enriched with 150 g fish/day (EPA 2-3 g). The haemostatic effects were determined as standardised skin bleeding times and their blood volumes, TXA<math>_2</math> formation <i>in vivo</i> in bleeding time blood and <i>in vitro</i> in clotting venous blood, platelet aggregability and platelet phospholipid fatty acid composition. The haemostatic changes by the diet were compared with those resulting from acetylsalicylic acid (ASA), a known inhibitor of TXA<math>_2</math> formation. The dietary fish increased the <math>\omega</math>-3 and decreased the <math>\omega</math>-6 fatty acids in platelet membrane phospholipids. It caused primary haemostasis to be delayed and platelet aggregability to be diminished similar to that caused by ASA but by a different mechanism. The diet caused no changes in the <i>in vivo</i> local formation of TXA<math>_2</math> contrasting to its effect in decreasing the <i>in vitro</i> formation in clotting venous blood. ASA on the other hand, greatly diminished the <i>in vivo</i> local production of TXA<math>_2</math>. During bleeding the amounts of <i>in vivo</i> TXA<math>_2</math> production were far smaller than those produced by the same volume of venous blood clotting <i>in vitro</i>. The findings of the present investigation indicate that addition of fish to the diet may have anti-thrombotic properties in man but also of augmenting any anti-thrombotic effect of ASA by dietary means. The haemostatic effects caused by the dietary fish cannot, however, be accounted for by diminished local production of TXA<math>_2</math> as commonly proposed, nor directly by decreased platelet aggregability or increase of <math>\omega</math>-3 fatty acids in the platelets. The available data also indicate that TXA<math>_2</math> is not the only or indeed the major determinant of skin bleeding time. The observations are compatible with the proposition that dietary fish alone or in combination with drugs may diminish, by some mechanism or other the incidence of arterial thrombosis in human properties.</p> |                                               |                            |
| <b>Key words</b> haemostasis, fish, eicosapentaenoic acid, $\omega$ -3 fatty acids, bleeding time, bleeding time blood volumes, thromboxane $A_2$ , platelets, platelet aggregation, arterial thrombosis, acetylsalicylic acid, phospholipids, prostaglandins.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                               |                            |
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*Margareta Thorngren*

Date

1983-10-06

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Indications of  
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Sweden

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# Contents

|                                                                                      |    |
|--------------------------------------------------------------------------------------|----|
| <b>Contents</b> .....                                                                | 5  |
| <b>Prephase</b> .....                                                                | 7  |
| <b>Background</b> .....                                                              | 9  |
| Development of the atherosclerotic lesion .....                                      | 9  |
| Pathogenesis of arterial thrombosis .....                                            | 9  |
| Platelets in haemostasis .....                                                       | 10 |
| Platelet and vascular prostaglandins .....                                           | 10 |
| Lipids and haemostasis in Greenland Eskimos .....                                    | 11 |
| <b>Aim of the present study</b> .....                                                | 15 |
| <b>Methods</b> .....                                                                 | 16 |
| Design of the study .....                                                            | 16 |
| Measurements of haemostatic effectiveness .....                                      | 17 |
| Bleeding time .....                                                                  | 17 |
| Bleeding time blood volumes .....                                                    | 17 |
| Thromboxane A <sub>2</sub> formation in the bleeding time blood <i>in vivo</i> ..... | 17 |
| Thromboxane A <sub>2</sub> formation in clotting venous blood <i>in vitro</i> .....  | 18 |
| Platelet aggregation .....                                                           | 18 |
| Lipid analysis .....                                                                 | 19 |
| <b>Results</b> .....                                                                 | 20 |
| Platelet and plasma lipids .....                                                     | 20 |
| Haemostatic parameters .....                                                         | 20 |
| Bleeding times .....                                                                 | 20 |
| Bleeding time blood volumes .....                                                    | 24 |
| Thromboxane A <sub>2</sub> formation in bleeding time blood <i>in vivo</i> .....     | 26 |
| Thromboxane A <sub>2</sub> formation in clotting venous blood <i>in vitro</i> .....  | 29 |
| Platelet aggregation .....                                                           | 29 |
| <b>General discussion</b> .....                                                      | 33 |
| <b>Concluding remarks</b> .....                                                      | 41 |
| <b>Acknowledgements</b> .....                                                        | 42 |
| <b>References</b> .....                                                              | 43 |



# Prephase

The present summary is based on the following papers, which will be referred to by their Roman numerals:

- I      Thorngren M, Gustafson A.**  
Effects of 11-week increase in dietary eicosapentaenoic acid on bleeding time, lipids, and platelet aggregation.  
Lancet 1981; ii: 1190–1193.
- II     Thorngren M, Gustafson A, Wohlfart G.**  
Effects of acetylsalicylic acid on platelet aggregation before and during increase in dietary eicosapentaenoic acid.  
Haemostasis 1983; 13: 244–247.
- III    Thorngren M, Shafi S, Born G.V.R.**  
Quantification of blood from skin bleeding time determinations: Effects of fish diet or acetylsalicylic acid.  
Haemostasis. In press.
- VI    Thorngren M, Shafi S, Born G.V.R.**  
Thromboxane  $A_2$  in skin bleeding time blood and in clotted venous blood before and after administration of acetylsalicylic acid.  
Lancet 1983; i: 1075–1078.
- V     Thorngren M, Shafi S, Born G.V.R.**  
Delay in primary haemostasis produced by a fish diet without change in local thromboxane  $A_2$ . Submitted.





# Background

The high incidence of *arteriosclerotic cardiovascular disease* and of its thromboembolic complications in western communities has stimulated and broadened research for new therapeutical strategies to prevent or moderate these conditions. The main focus remains on the physiopathology of the arterial wall and on its interaction with blood constituents. A number of recent experimental and clinical studies deals with the role of platelets in the development of atherosclerosis and of thromboembolic diseases (Ross and Glomseth 1976).

One current theory on *atherogenesis, the response-to-injury-hypothesis*, (Ross and Glomset, 1976; Ross, 1979) holds that arteriosclerotic plaque formation results from injury damaging the histological and functional integrity of the blood vessel endothelium. A number of causative factors have been proposed in endothelial damage. They include haemodynamic shearing at bifurcation of the arterial system, arterial hypertension, hypercholesterolemia with elevated concentrations of low-density lipoproteins (LDL) in the blood stream; smoking and other factors. It has been postulated that once the intact arterial endothelium is disrupted, plasma components especially the low density lipoproteins are deposited in the denuded area, followed by migration of smooth muscle cells into the subintimal space, where they proliferate. At the same time circulating platelets adhere-aggregate to seal off the injury with liberation of platelet thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and adenosine diphosphate (ADP) which causes further aggregation of adjacent platelets to the formation of a platelet plug, in what is to be interpreted as a chain reaction. Aggregating platelets release potent mitogenic substances, (platelet derived growth factors, PDGF), which stimulate further smooth muscle cells to proliferate and migrate into the intimal space. Formation of connective tissue matrix by the proliferated muscle cells and further deposition of intra and extra cellular lipids with secondary calcification gradually results in the advanced atherosclerotic plaque, which finally precipitates vascular occlusion.

Arteriosclerosis increases slowly, whereas *arterial thrombosis* occurs rapidly and is individual unpredictable. Arterial thrombi do not form in normal arteries and when formed in diseased vessels they consist initially of aggregated platelets. There is evidence that pathological aggregation of platelets in arteriosclerotic

arteries, such as the acute event of coronary thrombosis is initiated through fissures in underlying atheromatous plaques (Born, 1977; Davis, 1981). Platelets quickly adhere at the site of the vascular injury and then cohere to each other to build a thrombotic mass. Thus platelets have been claimed to play a major role not only in the development of the arteriosclerotic process but also in the clinical events of thromboembolic complications. Therefore, anything which diminishes the interaction between platelets and vessel walls is likely to limit the severity of the arteriosclerotic disease.

**Platelets** are important *in normal haemostasis*. The contribution of platelets to the haemostatic process involves a mechanism by which platelets adhere presumably to the sub-endothelial collagen in the vessel wall (Packham and Mustard, 1977) and a mechanism for the release of particular platelet constituents (i.e. ADP, prostaglandins) that accelerate further aggregation of adjacent platelets and trigger off coagulation of plasma to form a haemostatic plug.

Our knowledge on platelet-vessel wall interaction has widened during the last decade on the basis of the recent discoveries of the important modulators of platelet haemostasis, the **prostaglandins**, especially prostacyclin ( $\text{PGI}_2$ ) (Moncada et al, 1976a and 1977) and thromboxane  $\text{A}_2$  ( $\text{TXA}_2$ ) (Hamberg and Samuelsson, 1973; Hamberg et al, 1975). Prostaglandins are produced from the 20-carbon polyunsaturated fatty acids (PUFA) of membrane phospholipids, such as dihomogamma-linolenic acid (C 20:3) (DHLA), arachidonic acid (C 20:4) (AA) and eicosapentaenoic acid (C 20:5) (EPA), respectively precursors of the 1, 2, and 3 series, where arachidonic acid has been shown to have the greatest physiological importance (fig. 1). Arachidonic acid is released from membrane phospholipids by the action of a specific phospholipase  $\text{A}_2$  and converted by the enzyme cyclo-oxygenase to cyclic endoperoxides, which are rapidly transformed by thromboxane- and prostacyclin-synthetase to thromboxane  $\text{A}_2$  ( $\text{TXA}_2$ ) and prostacyclin  $\text{I}_2$  ( $\text{PGI}_2$ ) by platelets and vessel walls respectively.  $\text{PGI}_2$  is a potent vasodilator and inhibitor of platelet aggregation, whereas  $\text{TXA}_2$  causes platelets to aggregate and vessel walls to constrict. Both  $\text{TXA}_2$  and  $\text{PGI}_2$  formation can be inhibited by acetylsalicylic acid, (ASA) which acetylates the enzyme cyclo-oxygenase (Vane, 1971; Roth et al, 1975).

It has been suggested that the balance between  $\text{PGI}_2/\text{TXA}_2$  is an important mechanism for the haemostatic balance and arterial thrombosis tendency *in vivo* (Gryglewski et al, 1976; Moncada and Vane, 1981) and thus of importance in the atherosclerotic process. Numerous investigations have been undertaken to study this haemostatic concept and its therapeutical implications in thromboembolic disease, i.e. to find means to influence the  $\text{PGI}_2/\text{TXA}_2$  balance in the most beneficial way. Small doses of ASA have been suggested to have a favourable effect and therefore preferable in thromboembolic prophylaxis (Leading article, Lancet 1980) and thromboxane  $\text{A}_2$  synthetase inhibitors have been developed.

The extent to which physiological haemostasis and regulation of thrombus

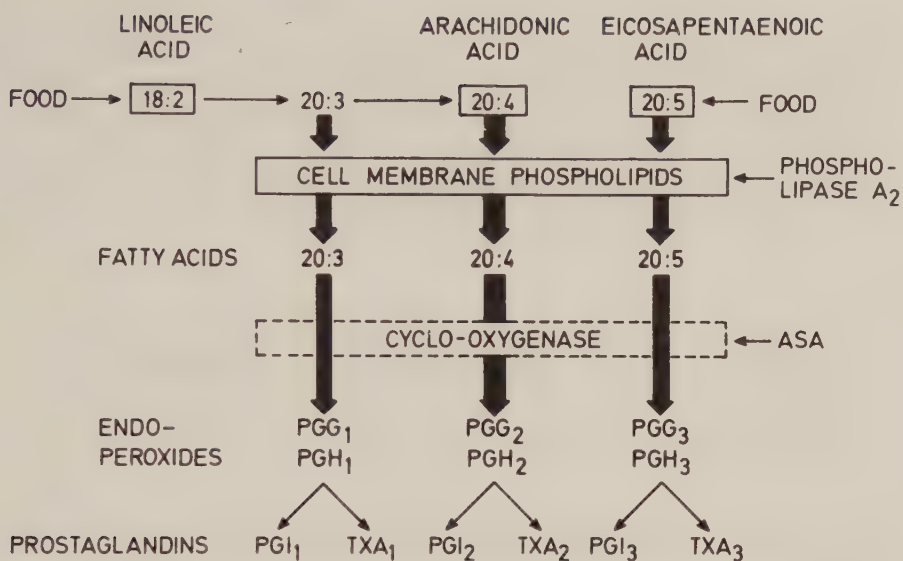


Fig. 1. Fatty acids in cell membrane phospholipids as precursors in prostaglandin synthesis.

formation *in vivo* really depends on prostaglandins is however still uncertain. Inhibition of the prostaglandin synthesis by ASA raises bleeding time by no more than about twice. There are many reports about the presence of thromboxane A<sub>2</sub> in circulating blood, and about its formation during aggregation of platelets or when blood clots *in vitro* (Lewy et al, 1979; Szczeklik et al, 1978; Patrono et al, 1980). However, the indicative value of those reports in evaluating the contribution of thromboxane A<sub>2</sub> to haemostasis at injury sites i.e. to the process of particular importance in which it is supposed to play a dominant role is questionable.

It has been suggested from epidemiological studies that atherosclerotic cardiovascular disease is uncommon among *Greenland Eskimos* (Bertelsen, 1940; Kronman and Green, 1980). The all over mortality from ischemic heart disease, i.h.d., in Greenlanders is said to average 3.5 % and in Danes 50 % (Dyerberg, 1981). Some observations indicate that this difference in morbidity pattern may be associated with differences in platelet-vessel wall interaction and has been attributed to differences in dietary habits (Dyerberg et al, 1978). The Eskimos have lower plasma cholesterol and LDL and much lower plasma triglycerides than do mainland Danes (Band and Dyerberg, 1972). These differences, documented in several studies as associated with lower risk of developing i.h.d., are not considered large enough to explain alone the difference in the mortality

from cardiovascular disease. The Eskimos also have in their plasma and platelet lipids low levels of long-chain polyunsaturated fatty acids of the  $\omega$ -6 family, i.e. arachidonic acid (AA), whereas the content of  $\omega$ -3 fatty acids, mainly eicosapentaenoic acid (EPA) is usually high; the source of these is the large amount of cold-water oily fish in the food (Dyerberg and Bang, 1979). EPA apparently originates far down the food-chain in phyto-plankton, which directly or indirectly serve as primary food source for all sea creatures.

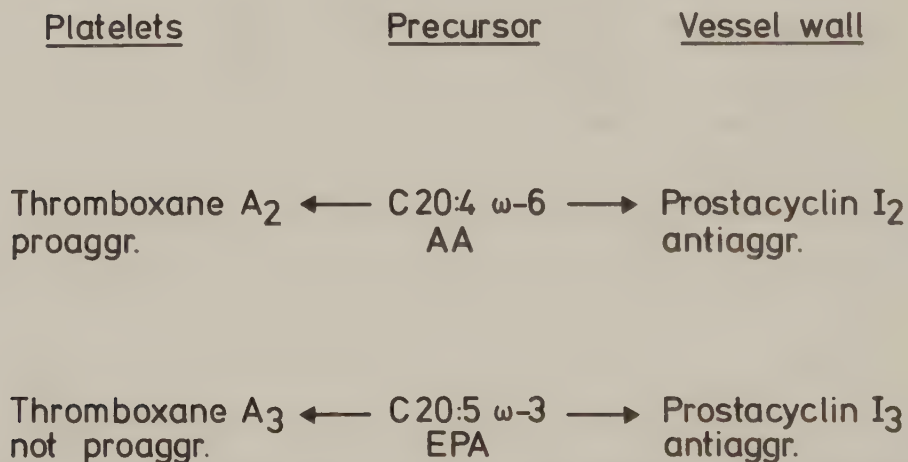


Fig. 2. Thromboxane and Prostacyclin formation from arachidonic acid (AA) and eicosapentaenoic acid (EPA), respectively.

These observations have led to a *hypothesis* which proposes that large quantities of  $\omega$ -3 polyunsaturated fatty acids, mainly EPA, in the diet protect the Eskimos against the thromboembolic complication of cardiovascular disease by reducing platelet aggregability (Dyerberg et al, 1978). Various mechanisms have been proposed to account for the *decreased platelet aggregability of people on diets rich in  $\omega$ -3 PUFA* (Sinclair, 1977; Dyerberg et al, 1978). Since fatty acids are precursors in the prostaglandin biosynthesis it has been suggested that EPA can use the vessel wall to make prostaglandin I<sub>3</sub> which is as potent as prostaglandin I<sub>2</sub> (prostacyclin) in inhibiting platelets, whereas the platelets themselves would from the EPA produce thromboxane A<sub>3</sub>, which unlike thromboxane A<sub>2</sub> has no aggregating effect (fig. 2). The anti-thrombotic condition brought about would then be due to an *imbalance between opposing prostaglandin derivatives* with the platelet inhibitory effect predominant. Dyerberg and Bang demonstrated impairment of haemostasis, i.e. prolongation of bleeding time and decreased



platelets aggregability in Eskimos compared to Danes (Dyerberg and Bang, 1979). Furthermore, *in vitro* experiments with EPA were shown to inhibit platelet aggregation presumably by a competitive inhibition of platelet cyclo-oxygenase (Dyerberg 1978).

## EPIDEMIOLOGICAL FINDINGS

1. The Eskimos almost lack cardiovascular disease.
2. Platelet aggregability is reduced.
3. Bleeding time is prolonged.
4. Different fatty acid composition in platelet and plasma.
  - a/ High proportion of  $\omega$ -3 fatty acids.
  - b/ Low proportion of  $\omega$ -6 fatty acids.
  - c/ Due to difference in diet.

Question I. Is number 1 a consequence of 2 + 3 + 4?  
II. Does 4 result in 2 and 3?

Fig. 3. Epidemiological and biochemical findings in Greenland Eskimos.

These epidemiological and biochemical findings raises *two questions* (fig. 3). **First**; is the low incidence of cardiovascular disease among Eskimos a consequence of reduced platelet aggregability due to dietary habits? **Secondly**; does long-term dietary enrichment with EPA prolong the bleeding time and reduce the aggregability of platelets and their production of thromboxane  $A_2$  due to related changes in the fatty acid composition of platelet lipids?

The traditional Eskimo diet of extraordinarily high intake of marine animals is too extreme to be acceptable by humans with different dietary habits. Against this background a diet rich in EPA and usable for long term studies was composed. In a pilot experiment the usual Swedish diet of two volunteers was enriched with EPA by merely replacing parts of the red meat by acceptable amounts of easily available fatty fish. This comparatively small change in the diet was sufficient to cause increases in the  $\omega$ -3 fatty acids of platelet phospholipids and to prolong the bleeding time, i.e. cause primary haemostasis to be delayed.

Based on these preliminary findings the present study was conducted in larger



groups to further elucidate possible connections between diets rich in  $\omega$ -3 fatty acids and haemostasis, and designed to give information on the proposed hypothetical mechanism behind such a connection, if any. During the work it became increasingly important to try to find out the extent to which primary haemostasis *in vivo* really depends on thromboxane A<sub>2</sub>.

# Aim of the study

- to study the effects, if any of a fish-enriched diet on haemostatic parameters:
  1. the primary haemostatic process, i.e. bleeding times
  2. bleeding time blood volumes as a function of time
  3. platelet aggregability *in vitro*
  4. thromboxane  $A_2$  formation *in vivo* and *in vitro*
  5. fatty acid composition of platelet and plasma lipids
- to relate any effects on haemostatic parameters by the fish diet to changes in platelet lipids
- to compare the haemostatic effects by the fish diet with those of a known inhibitor of prostaglandin synthesis, i.e. acetylsalicylic acid (ASA)
- to evaluate the proposed hypothesis that impairment of haemostasis by a fish diet is due to reduced thromboxane  $A_2$  formation
- to develop a technique for quantifying the blood emerging from standardised bleeding time skin incisions as a function of time
- to develop an *in vivo* technique for evaluating the contribution of thromboxane  $A_2$  to the physiological haemostatic process.

# Methods

## Design of the study

Two separate dietary investigations were performed, one year apart, in 10 and 12 apparently healthy male volunteers, respectively. The duration of the first dietary study was eleven weeks and that of the second six weeks. The subjects were male students in Lund and aged 21–39 years, all non-smokers. They had taken no drugs for at least two weeks before nor did they take any during the investigations.

*The diet:* For the study the volunteers took their normal Swedish diet but increased their intake of fish. They replaced according to personal taste especially red meat by approximately 150 gram per day of fatty fish, rich in  $\omega$ -3 fatty acids, such as herring, salmon and mackerel. Care was taken to use mostly fresh fish which was often consumed smoked, salted or raw. These dietary changes increased the EPA intake by 2–3 gram/day.

Tests of haemostasis and lipid analyses were made on two separate occasions a fortnight apart before the diet; after different times on the diet, and on at least two occasions after the diet. Samples were taken in the morning after an overnight fast of twelve hours.

Determinations were also made 2 hours after oral administration of a single dose of acetylsalicylic acid (ASA) 3.5 mg/kg and 10 mg/kg body weight during the baseline period of the first investigation (the effects of the two doses were determined two weeks apart) and after 11 weeks of the diet (when the higher dose was given 2 hours after the lower dose). During the control period of the second dietary study measurements were made in the same way after an ASA dose of 5 mg/kg body-weight.

In ten similar English subjects, bleeding times and thromboxane B<sub>2</sub> in bleeding time blood were determined on one occasion. Bleeding times were determined by the same observer in both the Swedish and the English volunteers.

Statistical evaluation of lipid analysis and of thromboxane B<sub>2</sub> concentrations in bleeding time blood and in clotted venous blood was by Student's t-test and of bleeding times, bleeding time blood volumes and platelet aggregation by Wilcoxon's test. Values are shown as mean  $\pm$  s.e.m.

# Measurements of haemostatic effectiveness

## Bleeding time

The bleeding time is defined as the time between the making of a small incision and the moment when bleeding stops. It is generally considered as the combined haemostatic response of small vessels, capillaries, arterioli and venules are cut together under standardised conditions. Bleeding times were determined by the method of Ivy (1940–41) as modified by Mielke et al, 1969. A blood pressure cuff was wrapped around the upper arm and inflated to 40 mm Hg. In an area free from visible veins just below the cubital fold two transverse incisions were made. Bleeding time was measured in duplicate, always by the same observer using the standardised Simplate II device from General Diagnostics. The Simplate II device (Babson and Babson, 1978) is designed to make two incisions, 5 mm long by 1 mm deep. The skin incisions were blotted every 30 seconds with a Whatman number 1 filter paper disc until bleeding stopped. The mean of the bleeding time after the two incisions was taken as the bleeding time.

## The bleeding time blood volumes

The bleeding time blood volumes were eluted from the filter paper as described in paper III, by placing each spot in 6 ml of Drabkins solution. Two hours later all blood was eluted from the filter paper. In each eluate the haemoglobin concentration was determined spectrophotometrically (Universal Photometer, Vitatron) and converted to corresponding blood volume from standard curves. The results were expressed as microliter of blood emerging in each 0.5 min period. To evaluate the completeness of elution of blood from the filter paper by the described procedure, known volumes of peripheral blood were put on filter paper, eluted and the haemoglobin concentration was compared with that of known volumes put directly into Drabkins solution. The correlation coefficient between these two procedures was highly significant ( $r=0.99$ ,  $n=40$ ). Platelets were counted in ultra flow 100 (Clay Adams).

## Thromboxane A<sub>2</sub> formation in the bleeding time blood *in vivo*

Thromboxane A<sub>2</sub> formation in the bleeding time blood *in vivo* (paper IV) were determined by radioimmunoassay of the stable degradation product thromboxane B<sub>2</sub>. The blood emerging from skin incisions made by the Simplate I device on the other arm was collected in one-minute periods into capillary tubes containing known volumes of a mixture of 100 mM EDTA and indomethacin at 5.6 mM to inhibit formation of thromboxane B<sub>2</sub> *in vitro*. After mixing the tubes were centrifuged at 15,000 g for 2 min. The clear supernatants were removed, frozen and stored at  $-20^{\circ}\text{C}$  and assayed later for thromboxane B<sub>2</sub>. The thromboxane B<sub>2</sub> standard (U51541 lot 0071-CLM-059C) and the antibodies against thromboxane

B<sub>2</sub> (21) were kindly supplied by Dr. John Pike of the Upjohn Company in Kalamazoo, U.S.A. and Dr. John Vane of the Wellcome Foundation Ltd., Beckenham, U.K., respectively. The total amounts of thromboxane B<sub>2</sub> formed in the bleeding time blood were calculated for each individual by summing the products of the concentrations in successive blood samples and their volumes.

In one of the volunteers the variability in the rate of the appearance of thromboxane B<sub>2</sub> in the bleeding time blood was determined. The mean standard error of 6 determinations was 0.1 ng/ml.

### **Thromboxane A<sub>2</sub> formation *in vitro***

Thromboxane A<sub>2</sub> formation *in vitro* was determined also in the serum from blood collected by venepuncture into glass tubes in which it was made to clot by incubation at 37°C for 1 h. The tubes were centrifuged at 2000 g for 10 min, the supernatant sera separated, frozen at -20°C and assayed for thromboxane B<sub>2</sub> by the same procedure.

### **Platelet aggregation**

Venous blood was allowed to flow into siliconized glass tubes containing 3.8 % trisodiumcitrat (proportion 9:1). It was centrifuged at 275 g at room temperature for ten minutes to produce platelet rich plasma (PRP). Platelet poor plasma (PPP) was prepared by re-centrifugation of the red cell fraction at 2,600 g for 20 minutes. Platelet aggregation in PRP in the presence of suspension of aggregating agents was measured photometrically (Born, 1962) under constant magnetic stirring and continuous automatic recording of transmission in a Payton aggregometer. In this apparatus 0.45 ml PRP was used, to which 50 µl of the aggregating agents were added. The PRP was not used within the first 30 minutes and the experiments were always finished within two hours (Seiss et al, 1981). The aggregating agents were collagen (Hormone-Chemie, Munich) and adenosinediphosphate (ADP) (SIGMA, St. Louis, U.S.A.), added at increasing concentration.

The indices of aggregation were (1) maximum decrease in optical density expressed as a percentage of the difference in optical density between platelet-rich and platelet-poor plasma; (2) maximum velocity arbitrarily expressed in mm/min; and (3) the lowest concentration of ADP which induced secondary aggregation, i.e. the release reaction. This concentration is referred to as the threshold value of ADP.



## Lipid analysis

Platelets and plasma were prepared for lipid analysis according to a method already described with minor modifications<sup>11</sup>. Extraction was with chloroform-methanol 2:1 or 1:1 for platelets and plasma respectively in the presence of butylated hydroxytoluene. Platelet phospholipids were separated by thin layer chromatography on 0.5 mm Silica gel with chloroform-methanol-acetic acid-water (65:24:4:4 v/v). The separated fraction containing phosphatidyl choline (PC) and including phosphatidyl inositol and phosphatidyl serine was methylated and analysed for fatty acids by gas-liquid chromatography on a Varian 3700 equipped with a flame-ionisation detector on a 200 cm glass column (2 mm internal diameter) packed with 15 % diethylene/glycol/succinate (DEGS) coated on chromosorb W, 80–100 mesh. The column was operated at 190° with nitrogen as carrier gas. Individual esters were identified by comparison with standards and of phospholipid fatty acid methylester from bull testis. Peaks were quantified and the fatty acid distributions estimated by a computer CDS 111 (with 17:0 heptadecanoic acid as internal standard).

# Results

## Platelet and plasma lipids (I, V)

The effects of the diet were similar in the two separate studies. The fish diet rich in  $\omega$ -3 fatty acids produced changes in the relative fatty acid composition of the phosphatidyl choline, i.e., lecithin, of plasma and platelets (Table 1). Already after one week the proportion of  $\omega$ -3 polyunsaturated fatty acids in both platelet and plasma phosphatidyl choline increased, due to an increase of both eicosapentaenoic acid (C 20:5), docosahexaenoic acid (C 22:6); these changes persisted throughout the diet. The proportion of  $\omega$ -6 fatty acids fell after one week in the platelets mainly due to a decrease in arachidonic acid (C 20:4) but also in linoleic acid (C 18:2); and in the plasma because of a considerable decrease in linoleic acid but after 3 weeks also in arachidonic acid. These changes resulted in a three-fold increase in the ratio of C 20:5/C 20:4 in the platelets. The effects of the diet on the changes in the proportions of the polyunsaturated fatty acids were similar in plasma and platelets (Table 1). Three weeks after the end of the diet the fatty acid composition of both plasma and platelet PC had reverted almost entirely to the pre-dietary pattern.

## Haemostatic parameters

### Bleeding times (I, III, IV, V)

Before dieting began the mean bleeding times were not significantly different between the two separate dietary studies. The mean bleeding time for the Swedish volunteers was  $8.4 \pm 0.4$ . In ten similar English subjects the bleeding time was  $5.6 \pm 0.4$  min; this was much shorter than the bleeding times in the Swedish subjects, the difference being highly significant ( $p < 0.001$ ).

### Effects of dietary fish

The fish diet produced a prolongation of bleeding time but the onset of this effect varied between the two separate investigations. Bleeding time was not increased significantly one and three weeks on the second dietary period (fig. 4) although increased at three weeks by 31 % during the first. After six weeks in both dietary investigations the bleeding time increased in most volunteers by a mean of 40 % and was prolonged throughout the dietary experiments. Although there were considerable differences between individuals (range: 6–93 %) the mean increase was highly significant ( $p < 0.01$ ). Three weeks after the end of the diet, the bleeding time was still increased; it was a little prolonged at six weeks, although not significantly, but no longer after eight and sixteen weeks on the normal diet.

There were no significant changes in the concentration of platelets in peripheral blood during the diet compared with before and after.

#### Platelet lipid composition

|                     |      |      |      |      |      |      |
|---------------------|------|------|------|------|------|------|
| C 20 : 5            | 0.6  | 1.7  | 1.7  | 2.0  | 0.9  | 0.9  |
| C 20 : 4            | 18.3 | 17.0 | 16.3 | 15.6 | 17.2 | 18.0 |
| Ratio $\times 10^3$ | 33   | 100  | 104  | 128  | 52   | 50   |

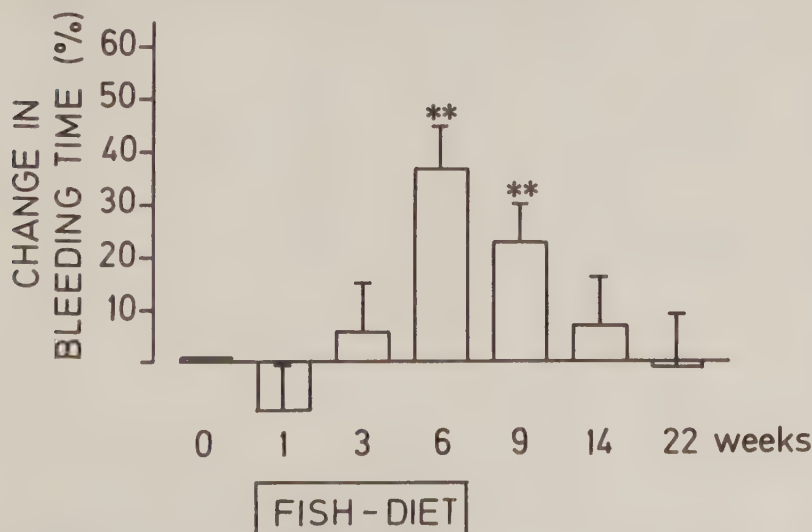


Fig. 4. Effect of the fish-diet on *in-vivo* bleeding time and platelet lipid composition.

\*  $p < 0.05$ ; \*\*  $p < 0.01$

Table 1 Fatty acid composition (per cent) in platelet phosphatidyl choline (PC) before, during and after the fish diet.

|                                                                      | 16:0           | 18:0             | 18:1            | 18:2            | 20:3            | 20:4             | 20:5            | 22:5            | 22:6            | Total           | Total            |
|----------------------------------------------------------------------|----------------|------------------|-----------------|-----------------|-----------------|------------------|-----------------|-----------------|-----------------|-----------------|------------------|
|                                                                      |                |                  |                 | $\omega$ -6     | $\omega$ -6     | $\omega$ -6      | $\omega$ -3     | $\omega$ -3     | $\omega$ -3     | $\omega$ -6     | $\omega$ -6      |
| <i>Baseline</i>                                                      |                |                  |                 |                 |                 |                  |                 |                 |                 |                 |                  |
| 0 weeks                                                              | 19.9<br>(0.6)  | 22.7<br>(0.2)    | 22.6<br>(0.2)   | 7.0<br>(0.2)    | 1.6<br>(0.1)    | 18.3<br>(0.4)    | 0.6<br>(0.1)    | 0.9<br>(0.1)    | 1.5<br>(0.1)    | 4.3<br>(0.2)    | 28.9<br>(0.5)    |
| <i>Dietary period</i>                                                |                |                  |                 |                 |                 |                  |                 |                 |                 |                 |                  |
| 1 weeks                                                              | 20.4<br>(0.3)  | 22.2**<br>(0.2)  | 22.5<br>(0.2)   | 6.1***<br>(0.2) | 1.4*<br>(0.1)   | 17.0**<br>(0.3)  | 1.7***<br>(0.2) | 1.0***<br>(0.1) | 2.1***<br>(0.2) | 6.4***<br>(0.4) | 26.2***<br>(0.5) |
| 3 weeks                                                              | 20.8<br>(0.3)  | 21.9***<br>(0.2) | 22.9<br>(0.2)   | 6.5***<br>(0.2) | 1.3***<br>(0.1) | 16.3***<br>(0.4) | 1.7***<br>(0.1) | 1.1***<br>(0.1) | 2.3***<br>(0.2) | 6.5***<br>(0.3) | 25.8***<br>(0.4) |
| 6 weeks                                                              | 20.9*<br>(0.2) | 21.7***<br>(0.3) | 23.4**<br>(0.2) | 6.2***<br>(0.2) | 1.3***<br>(0.1) | 15.6***<br>(0.4) | 2.0***<br>(0.2) | 1.1***<br>(0.1) | 2.5***<br>(0.2) | 7.2***<br>(0.3) | 24.6***<br>(0.5) |
| <i>Post dietary period</i>                                           |                |                  |                 |                 |                 |                  |                 |                 |                 |                 |                  |
| 3 weeks                                                              | 20.0<br>(0.3)  | 22.4**<br>(0.3)  | 22.9<br>(0.2)   | 7.1<br>(0.2)    | 1.6<br>(0.1)    | 17.2*<br>(0.4)   | 0.9***<br>(0.1) | 1.0**<br>(0.1)  | 1.9***<br>(0.1) | 5.1***<br>(0.2) | 27.8*<br>(0.4)   |
| 8 weeks                                                              | 19.9<br>(0.3)  | 22.7<br>(0.3)    | 22.8<br>(0.2)   | 6.7<br>(0.3)    | 1.6<br>(0.1)    | 18.0<br>(0.4)    | 0.9**<br>(0.1)  | 0.9<br>(0.1)    | 1.8**<br>(0.2)  | 4.8*<br>(0.3)   | 28.1<br>(0.5)    |
| Correlation co-efficient with corresponding values for plasma PC are | 0.22*          | 0.40***          | 0.04            | 0.71***         | 0.44***         | 0.57***          | 0.92***         | 0.33***         | 0.65***         | 0.84***         | 0.83***          |

Upper values are means, lower values s.e.m. Significance of differences

\* p &lt; 0.05    \*\* p &lt; 0.01    \*\*\* p &lt; 0.001

*Effects of acetylsalicylic acid (ASA)*

Before dieting began ASA at doses of 3.5 mg/kg and of 10 mg/kg body-weight prolonged bleeding time by 29 % ( $p < 0.05$ ) and 41 % ( $p < 0.01$ ) respectively, i.e. by as much as did the diet alone (fig. 5). ASA 5 mg/kg body-weight prolonged bleeding time by 124 % ( $p < 0.01$ ). At the end of the dietary period ASA increased the already prolonged bleeding time by 55 % ( $p < 0.01$ ) and 44 % ( $p < 0.05$ ) after doses of 3.5 and 10 mg/kg body-weight respectively.

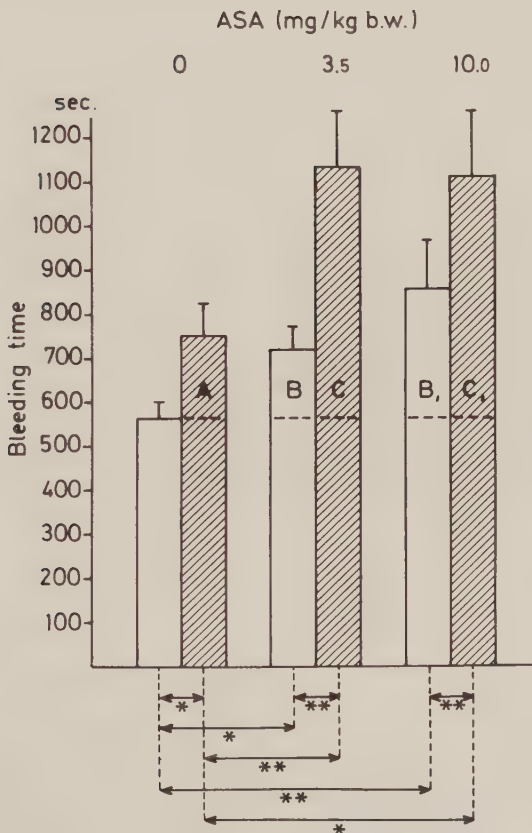


Fig. 5. Effect of acetylsalicylic acid (ASA) on bleeding time before (open column) and during 11th week of (hatched column) EPA-rich diet.

\*  $p < 0.05$ ; \*\*  $p < 0.01$



### Bleeding time blood volumes (III)

In the control period the volume of blood emerging from the cuts increased in the first two minutes irrespective of the final bleeding time; and then decreased continuously until bleeding stopped (fig. 6). In the nineteen volunteers the total bleeding time blood volumes was  $505 \pm 75 \mu\text{l}$ . The total volumes of blood emerging from the time of incisions up to the maximum as well as total blood volumes were significantly correlated with the corresponding bleeding times ( $r=0.77$ ,  $p<0.001$ ) and ( $r=0.66$ ,  $p<0.01$ ) respectively.

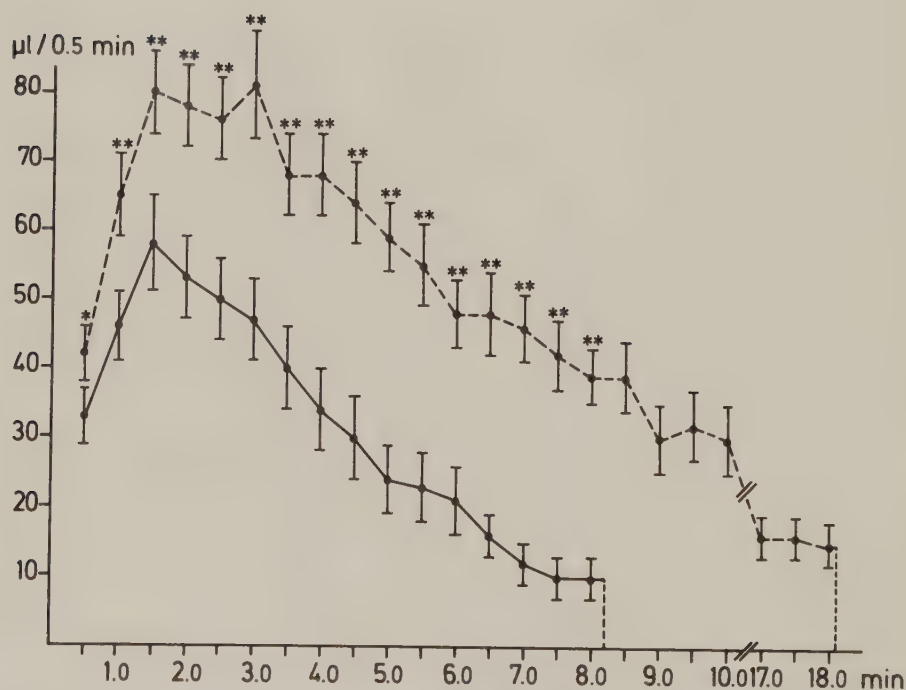


Fig. 6. Blood loss ( $\mu\text{l}/0.5 \text{ min}$ ) from skin bleeding time determinations (on 19 subjects) as a function of time (min) under control conditions ( $\bullet$ — $\bullet$ ) and after administration of ASA (5 mg/kg body-weight) ( $\bullet$ — — — $\bullet$ ).

\*\*  $p < 0.01$

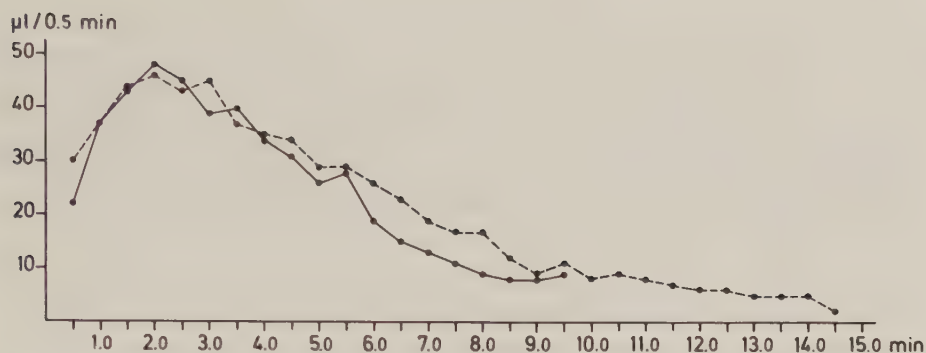


Fig. 7. Blood loss ( $\mu\text{l}/0.5 \text{ min}$ ) from skin bleeding time determinations as a function of time (min) for one of the volunteers during control condition (●—●) and after 6 weeks (●— — —●) on the fish supplement diet.

### *Effects of dietary fish*

After six weeks on the diet, when bleeding times were increased, the bleeding time blood loss again increased to a maximum after about two minutes and then decreased progressively until 2–3 minutes before bleeding ceased; during that final period blood continued to emerge in small quantities (fig. 7). The correlation between bleeding time and blood loss up to the two minute maximum persisted, although less significantly ( $r=0.48$ ,  $p<0.05$ ). During the diet the rate of blood loss was not significantly different from controls, except for at three weeks when it was significantly less than before the diet ( $p<0.01$ ).

### *Effects of Acetylsalicylic Acid (ASA)*

When nineteen volunteers took one dose of ASA 5 mg/kg b.w. their total bleeding time blood volumes increased to  $1400 \pm 135 \mu\text{l}$ . These total blood volumes were still correlated with the corresponding bleeding times ( $r=0.67$ ,  $p<0.001$ ), but there was no longer a correlation between the bleeding time and the blood loss up to its maximum ( $r=-0.16$ ). The blood loss versus time curve was similar to that before but the quantity of blood lost was significantly greater throughout ( $p<0.01$ ) (fig. 6).

### Thromboxane A<sub>2</sub> formation in bleeding time blood (IV, V)

In eleven Swedish volunteers the bleeding time blood contained thromboxane B<sub>2</sub> in concentrations which increased almost linearly ( $r=0.69$ ,  $p<0.001$ ) from the first to the fifth minute, both in the groups as a whole (fig. 8) and in most individuals. There were considerable differences in the thromboxane B<sub>2</sub> concentration between individuals. After 5 min the amount of blood coming from the cuts was too small for accurate quantification and thromboxane B<sub>2</sub> assay.

The thromboxane B<sub>2</sub> concentration in the bleeding time blood of the six volunteers who had the longest bleeding time was then compared with that of the 5 volunteers who had shorter bleeding times. The individuals, whose bleeding times were less than 7.5 min, had significantly ( $p<0.05$ ) higher concentrations of thromboxane B<sub>2</sub> at most intervals in the bleeding time blood than those who had longer bleeding times.

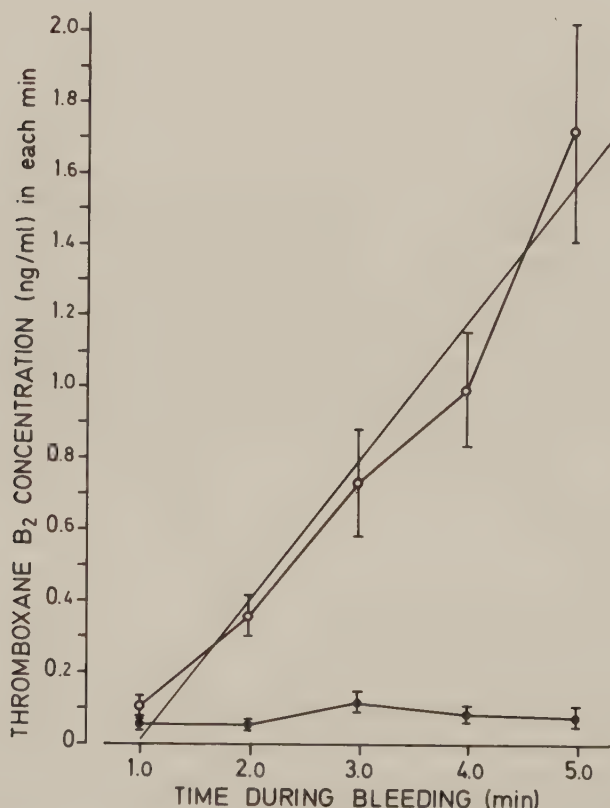


Fig. 8. Thromboxane B<sub>2</sub> concentrations in bleeding time blood before (○—○) and after (●—●) one dose 5 mg/kg b.w. of ASA.

In the English volunteers the increase in thromboxane B<sub>2</sub> concentration in the bleeding time blood with time was similar to that of the Swedish volunteers, but the concentrations were significantly greater ( $p < 0.05$ ) at all times.

The total amount of thromboxane B<sub>2</sub> produced per minute in the bleeding time blood increased progressively during the first three minutes and was thereafter constant for as long as assays were possible (fig. 9). There was no correlation between the production rate of thromboxane B<sub>2</sub> and the bleeding time ( $r = 0.02$ ).

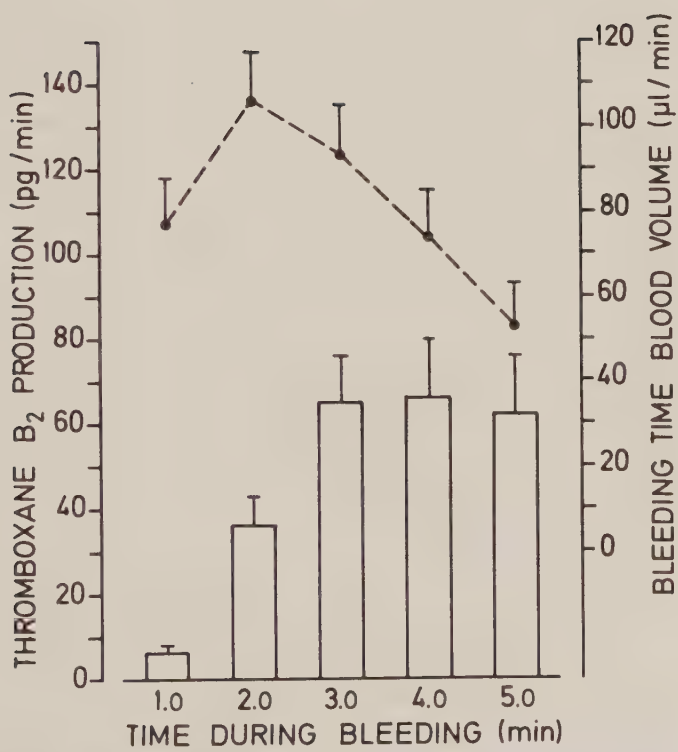


Fig. 9. Rate of thromboxane B<sub>2</sub> production in blood coming from bleeding time skin incisions (Simplate II) of 11 Swedish volunteers in successive one-minute period.

### *Effects of dietary fish*

After one and three weeks on the fish diet; when the bleeding time was not yet prolonged there were no significant changes in the thromboxane  $B_2$  concentrations in the bleeding time blood (fig. 10). After six weeks when the bleeding time was prolonged there were still no changes in the thromboxane  $B_2$  concentrations nor again 3 and 8 weeks after the end of the diet (fig. 10). The total amount of thromboxane  $B_2$  produced per minute in the bleeding time blood and was also unchanged during the diet. Even in the six individuals whose bleeding times were increased most by the diet, the rate of thromboxane  $B_2$  production was not significantly changed.

### *Effect of Acetylsalicylic Acid (ASA)*

When the Swedish volunteers took a single dose of ASA (5 mg/kg b.w.) thromboxane  $B_2$  concentrations in the bleeding time blood were greatly diminished, but small, measurable concentrations persisted which did not increase with time (fig. 8).

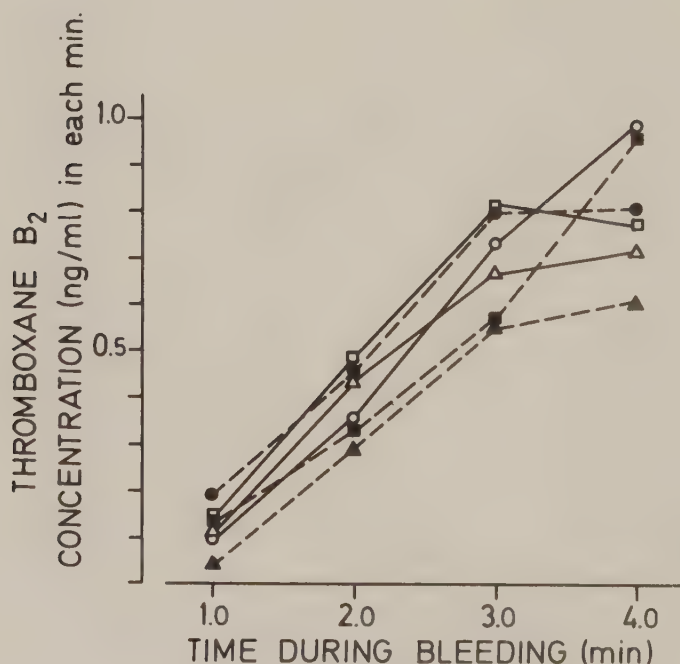


Fig. 10. Thromboxane  $B_2$  concentrations in the bleeding time blood before (○—○) and after 1 (▲---▲), 3 (■---■) and 6 weeks (●---●) of the fish-diet and 3 (△—△) and 8 weeks (□—□) after the end of the diet.



### **Thromboxane A<sub>2</sub> formation in clotting venous blood (IV, V)**

In the Swedish volunteers, clotted venous blood produced  $168 \pm 23$  ng thromboxane B<sub>2</sub>/ml. There was no relation between the bleeding time and the concentration of thromboxane B<sub>2</sub> in clotting venous blood.

To evaluate the relevance, if any, of thromboxane A<sub>2</sub> concentrations in clotted venous blood to the appearance of thromboxane A<sub>2</sub> in blood from a haemostatic site, the total amounts of thromboxane B<sub>2</sub> formed in bleeding time blood during the first five minutes were calculated for each individual by summing the products of the concentration in successive blood samples and their volumes. The total amounts of thromboxane B<sub>2</sub> were also calculated for the same volumes of blood clotting *in vitro*. These comparisons demonstrated that the amount of thromboxane A<sub>2</sub> formed by blood clotting *in vitro* ( $74.8 \pm 12.3$  ng) exceeded by two orders of magnitude that produced by the same volume of blood during haemostasis in skin incisions *in vivo* ( $0.23 \pm 0.03$  ng).

#### *Effects of dietary fish*

After one week of the diet, the concentration of thromboxane B<sub>2</sub> in clotted venous blood was already significantly decreased ( $121 \pm 23$  ng/ml) and remained decreased during and for at least eight weeks after the diet.

#### *Effects of Acetylsalicylic Acid (ASA)*

After a single dose of ASA (5 mg/kg b.w.) the concentration of thromboxane B<sub>2</sub> in clotted venous blood *in vitro* was reduced to  $0.59 \pm 0.03$  ng/ml.

### **Platelet aggregation (I, V)**

#### *Effects of dietary fish*

The reduction by the dietary fish on platelet aggregation were similar in the two investigations. The aggregability of the platelets dropped after the start of the diet whether aggregation was induced by collagen or ADP.

Aggregation by collagen returned to predietary values while the volunteers were still on the diet except for the highest concentration ( $10 \mu\text{l/ml}$ ) of collagen for which it remained decreased until the end of the diet.

The lowest concentration of ADP required to induce the second phase of aggregation i.e. ADP threshold, was raised after one week on the diet. This evidence of decrease in platelet aggregability remained for the duration of the diet and at least six weeks thereafter. Aggregation by ADP measured as maximal velocity decreased after three weeks and remained decreased not only during the diet but for at least 16 weeks after its end (fig. 11). Platelet aggregability measured as maximum optical density decreased similarly.

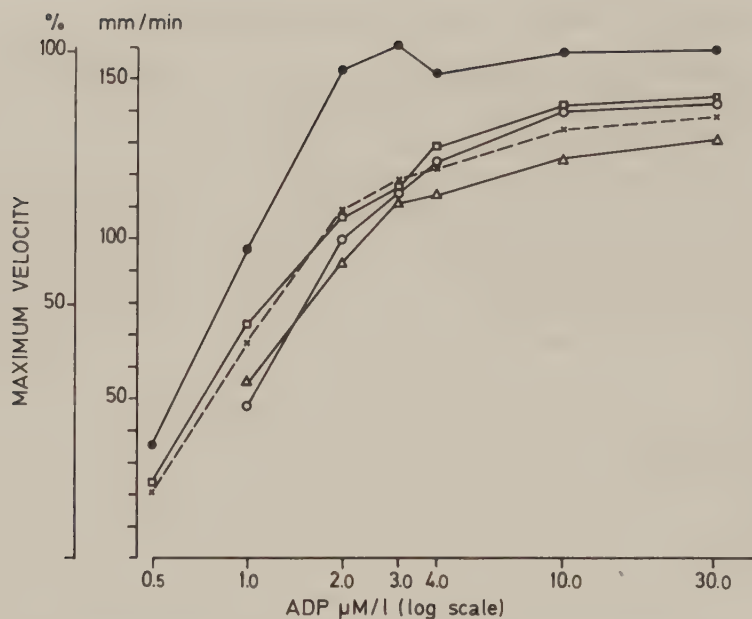


Fig. 11. Platelet aggregation (mean maximum velocity) by ADP before (●—●), after 1 (○—○), 3 (△—△) and 6 weeks (□—□) of the fish-diet and 16 weeks (×—×) after the end of the diet.

### *Effects of Acetylsalicylic Acid (ASA)*

**Collagen:** During the control period 3.5 mg/kg b.w. ASA decreased platelet aggregation by collagen. There was no further decrease after ASA 10 mg/kg b.w. At the end of the dietary period ASA decreased platelet aggregation to the same extent as during the baseline period (fig. 12).

**ADP:** In the control period the lowest concentration of ADP required to induce the second phase of aggregation, i.e. ADP threshold, was  $2.5 \pm 0.3 \mu\text{mol}$  (fig. 13). ASA 3.5 mg/kg b.w. increased the ADP threshold to  $4.4 \pm 0.2$  and 10 mg/kg b.w. to  $4.6 \pm 0.5$ , i.e. by as much as did the diet alone (fig. 13). At the end of the dietary period ASA increased further the ADP threshold to  $5.8 \pm 0.7$  and  $6.7 \pm 0.7 \mu\text{mol}$  after 3.5 and 10 mg/kg b.w. respectively.

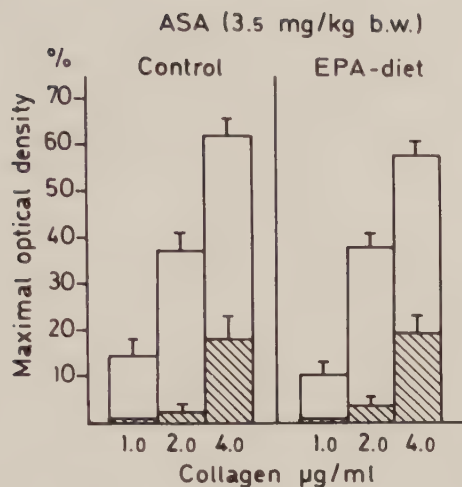


Fig. 12. Effect of ASA on platelet aggregation by collagen before and during the 11th week of EPA-rich diet.

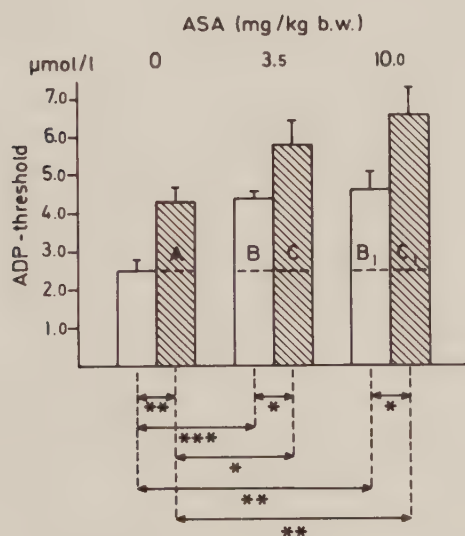


Fig. 13. Effect of ASA on platelet aggregability by ADP before, measured as ADP threshold, before (open column) and during the 11th week (hatched column) of EPA-rich diet.

\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$



# General discussion

Great interest has been aroused by the claim that the clinical manifestations of arteriosclerotic vascular disease are rare in Greenland Eskimos as well as in attempts to establish possible reasons for this (Kronman and Green, 1980). A *major hypothesis* proposes that large quantities of fish and marine mammals and thereby extraordinarily high content of  $\omega$ -3 polyunsaturated fatty acids, mainly eicosapentaenoic acid (EPA) in the diet protects the Eskimos against cardiovascular disease by reducing platelet aggregability (Dyerberg et al, 1978). According to the same hypothesis, long term dietary enrichment with EPA should prolong bleeding time because of reduced platelet aggregability which in turn should be related to changes in the fatty acid composition of platelets, whereby their production of the proaggregatory prostaglandin derivate, thromboxane  $A_2$  is diminished.

*The purpose of the present investigation* was to further elucidate possible effects in man of diets rich in  $\omega$ -3 fatty acids on haemostasis (I-III, V), of which there were no reports at the start of the study. Earlier investigations consisted mainly of epidemiological surveys of Greenland Eskimos (Bang and Dyerberg, 1972; Dyerberg et al, 1975; Dyerberg and Bang, 1978) or studied *in vitro* effects of isolated fatty acids on platelet properties (Dyerberg et al, 1978; Gryglewski et al, 1979). This investigation was conducted to find out whether the dietary enrichment with eicosapentaenoic acid by the addition of appropriate amounts of easy available fatty fishes would result in haemostatic changes in man, and if so, to relate any effects on haemostatic parameters to changes in platelet lipids.

Healthy young men were maintained for several weeks on their normal Swedish diet, the only change being an intake of approximately 150 g of fish per day – mainly mackerel, herring and salmon. This diet was well tolerated and increased the eicosapentaenoic acids (EPA) intake by 2–3 g per day. This is considerably more than would have been consumed in the average Swedish diet, but considerably lower than the daily consumption of the average Greenland Eskimo which according to Dr Dyerberg is equivalent to 5–6 g eicosapentaenoic acids per day (Dyerberg, 1981). Mindful of the proposed mechanism by which eicosapentaenoic acid would influence platelets/vessel wall interaction it was also of



interest to evaluate and relate possible haemostatic changes resulting from the diet with those resulting from acetylsalicylic acid, (ASA), a known inhibitor of thromboxane A<sub>2</sub> production, which is under the investigation for the prevention of thromboembolic complications of cardiovascular disease (The Canadian Co-operative study, 1978; Leading article Lancet, 1979; Lewis et al, 1983).

The given amount of fish in the diet was sufficient to produce the expected changes in the relative *fatty acid composition* of the platelet membrane phospholipids (I, V). Already after one week there were increases in the polyunsaturated fatty acids belonging to the  $\omega$ -3 series (i.e. C 20:5 and C 22:6) and decreases in the fatty acids of the  $\omega$ -6 series (mainly due to decreases in the arachidonic C 20:4 but also in linoleic acid C 18:2). These changes persisted throughout the diet and reverted to pre-dietary pattern three weeks after its end. The effects of the diet on the changes in platelet membrane fatty acids resulted in a three-fold increase in the ratio of C 20:5 to C 20:4 (i.e. EPA/AA) in the platelets, but not nearly to the extent found in platelets from Prof. Sinclair, who went on a diet strictly limited to marine animal food with water (Sinclair, 1981) or in platelets from Greenland Eskimos (Dyerberg and Bang 1979). Nevertheless, the comparatively small dietary changes in the present investigation caused primary haemostasis to be delayed and platelet aggregability to be diminished (I–III, V). These observations were in accordance with the prolonged bleeding time and the low aggregability of platelets from Greenland Eskimos who live largely on fish and therefore compatible with the proposition that marine fatty acids diminishes the incidence of arterial thrombosis in these populations (Sinclair, 1977; Dyerberg et al, 1978; Dyerberg and Bang, 1979).

The effects of the fish diet on the changes in the fatty acid composition of platelets were, however, not matched by the changes that occurred in the haemostatic parameters (I–III, V). The dietary fish produced *prolongation of the skin bleeding time* in the same proportion in both dietary investigations but the onset of this delay in the primary haemostasis process came later than the induced changes in the fatty acid composition of the platelets. Bleeding time was unaffected one week on the diet when the platelet lipid changes had already occurred. Although prolonged at three weeks during the first dietary investigation, the skin bleeding time was not definitely increased until six weeks on the diet in most volunteers by a mean of 40 %. Furthermore, bleeding time was still prolonged three weeks after the end of the diet when fatty acid composition of the platelets were back to normal. Large differences in bleeding time prolongation in different individuals were associated with small differences in fatty acid composition. Therefore the effects of the diet on the fatty acid composition of the platelets did not accompany its effect on the *in vivo* bleeding time.

The effects of the diet on the aggregability of platelets also failed to follow the changes in their fatty acid composition. The *aggregability of platelets dropped* whether aggregation was induced by collagen or ADP, which was compatible

with the previous hypothesis proposed to explain the bleeding time increase. However, it could not explain that the reactivity to collagen became normal again well before the end of the diet, whereas the aggregation by ADP was decreased throughout the diet and for several weeks thereafter; i.e. even when the platelet fatty acid composition had reverted to pre-dietary pattern and long after the normalization of the bleeding time. Since the effects of the EPA-rich fish diet on platelet membrane phospholipids did not parallel its effect on *in vitro* platelet aggregation, with a further divergence according to whether aggregation was induced by collagen or by ADP, it appears unlikely that the decreased platelet aggregability is directly due to changes in their fatty acid composition. Parallel investigations on fish oil supplements in the diet also indicate no consistent effect on platelet aggregation by collagen (Goodnight et al, 1981; Sanders and Roshanai, 1983) whereas platelet aggregation by ADP is decreased in human volunteers given salmon oil (Goodnight et al, 1981) as well as in Eskimos (Dyerberg and Bang, 1979) and in Japanese fishermen (Hirai et al, 1980).

Thus, the effects of the fish diet on the fatty acid composition of the platelets did not accompany its effect on either *in vivo* bleeding time or on *in vitro* platelet aggregation. These results make it unlikely that the increase in bleeding time depends directly on decreased platelet aggregability or directly on changes in their  $\omega$ -3 fatty acids and they also raise the possibility that as yet unidentified constituent(s) of fish diet might be responsible for the haemostatic abnormalities. This possibility is supported also by observations that dietary intake of increasing amounts of EPA by fish oil (Sanders et al, 1983) or EPA-concentrate (Dyerberg, 1981) did not increase the bleeding time further and certainly not in a dose dependent manner. The most pronounced bleeding time prolongation was achieved in the present investigation and in that of Goodnight et al (1981), in both of which meat of fish was used as supplement in the diet. On the other hand, despite the source of supplementation, bleeding time prolongations have been reported significant in most investigations with eicosapentaenoic acid in humans (Goodnight et al, 1981; Sanders et al, 1981; Lorenz et al, 1983; Dyerberg, 1981; Saynor et al, 1983) although exceptions exist (Bronx et al, 1981; Nagakawa et al, 1983).

The haemostatic changes resulting from the diet were compared with those resulting from *acetylsalicylic acid* (ASA) in the same individuals (I, II). ASA is thought to inhibit secondary aggregation and the release reaction by acetylating the cyclo-oxygenase of platelets and thereby preventing them from synthesizing the aggregating agent thromboxane  $A_2$  (Smith and Willis, 1971; Roth et al, 1975). ASA is well known to prolong bleeding time (Mielke et al, 1969) and to reduce platelet aggregation and is under the investigation for the prevention of thromboembolic complications of cardiovascular disease (Leading article Lancet, 1979; The Canadian Co-operative study group, 1978; Lewis et al, 1983). When given before the fish diet ASA, in doses which are shown to inhibit prostaglandin

synthesis almost completely (Preston et al, 1981) prolonged bleeding time and decreased platelet aggregability by ADP by as much as did the diet alone, and when given during the diet its effect was additive. Interesting is also the observation that when ASA was given during the diet, aggregation by collagen, which involves thromboxane  $A_2$  (Hamberg et al, 1975), decreased to the same extent as during the baseline period.

These results indicate that the *mechanism* by which a fish diet delays primary haemostasis is different from that of ASA, notwithstanding their apparently similar effect on bleeding time and platelet aggregation. This raises the possibility that dietary fish may have anti-thrombotic properties in man different from that of ASA (Lewis et al, 1983), but also of augmenting any anti-thrombotic effect of ASA by dietary means. Indeed, from animal work there is evidence for a protective effect of fish oils on experimental cerebral and myocardial infarction and on experimental thrombosis of vascular prosthesis (Black et al, 1979; Culp et al, 1980; Hornstra, 1982). The present findings also suggest that dietary fish does not delay haemostasis by reducing prostaglandin synthesis or causing an imbalance between pro and antiaggregatory prostaglandin derivatives.

There are other good reasons for doubting that an imbalance between pro and antiaggregatory prostaglandin derivatives is really likely to account for the prolongation of bleeding time by dietary fish. Thromboxane  $A_2$  is a very potent aggregating agent and it is produced *in vitro* in large quantities by aggregating platelets. If this occurs also *in vivo* it seems improbable that a fairly small decrease in thromboxane  $A_2$  production would diminish haemostatic platelet aggregation sufficiently to produce the observed increase in bleeding time.

As part of our attempt to find out by what mechanism(s) the dietary intake of fish increases the bleeding time *two novel techniques* for evaluating the primary haemostatic process were developed (III, V). The extent to which the physiological haemostasis really depends on thromboxane  $A_2$  and related endogenous agents is still uncertain. There are many reports about the presence of thromboxane  $A_2$  in circulating blood (Lewy et al, 1979) and about its formation during aggregation of platelet-rich plasma (Szczeklik et al, 1978; Seiss et al, 1981) or when blood clots *in vitro* (Patrono et al, 1980) and how this is affected by drugs (Patrono et al, 1980) or recently also by diets (Dyerberg, 1980; Seiss et al, 1980; Terano et al, 1983). However, the relevance, if any of those reports in evaluating the contribution of thromboxane  $A_2$  to haemostasis at injury sites — i.e. in the process in which it is supposed to play a dominant role — is questionable.

To determine the *contribution of thromboxane  $A_2$*  to primary haemostasis of healthy human beings *in vivo*, under normal conditions (IV) and in particular after dietary induced prolongation of the bleeding time (V), thromboxane  $A_2$  was measured as its stable metabolite thromboxane  $B_2$  in blood coming from standard incisions made for the skin bleeding time and during *in vitro* clotting of venous blood from the same individuals.



Determination of the *skin bleeding time* by the Simplate technique is the most reproducible parameter of haemostatic effectiveness currently in clinical use (Mielke et al, 1969; Babson and Babson, 1978). Like other bleeding times, it is a measure of the duration of bleeding from the moment of injury to the complete cessation of bleeding. The technique provides no information about the pattern of blood loss from the incision nor how this may be affected by different physiological or pathological conditions or by pharmacological agents. The Simplate II technique for measuring bleeding time was also adapted to *quantify the volume of blood* as a function of time (III) and for determining how this parameter was affected by either acetylsalicylic acid (ASA) or the dietary supplement of fish. The recovery of the emerging blood also permitted the estimation of the total amounts of thromboxane B<sub>2</sub> formed in the bleeding time blood by summing the products of the thromboxane B<sub>2</sub> concentration in successive blood samples and their volumes.

In the blood coming from the *skin incisions* standardised for the bleeding time determinations, *thromboxane A<sub>2</sub> concentrations* increased linearly for as long as enough blood emerged for the determinations, but the amounts of thromboxane A<sub>2</sub> produced were far smaller than those produced by the same volume of venous blood clotting *in vitro* (IV). The English subjects had shorter bleeding times and more thromboxane A<sub>2</sub> in the bleeding-time blood than the Swedish subjects. When the Swedish subjects were grouped according to bleeding times those with the shortest had higher thromboxane A<sub>2</sub> concentrations than those with the longer bleeding times. Both findings indicate a connection between the duration of bleeding and the capacity of the local haemostatic mechanism, presumably the plug of platelets to produce thromboxane A<sub>2</sub>. Nevertheless, they are also compatible with the possibility that thromboxane A<sub>2</sub> is a measure of the amount of platelet aggregation rather than its cause.

The *rate of blood loss* from the standard skin incisions rose for about 2 min, after which it fell continuously until bleeding stopped (III, IV). This increase is explained most simply on the assumption that the incision causes rapid vasoconstriction immediately about it, which is reversed in the subsequent two minutes or so. This pattern occurs regardless of the final bleeding time, and the quantity of blood emerging from the time of incision up to the maximum is correlated significantly with the final bleeding time.

It is apparent that thromboxane B<sub>2</sub> concentrations can rise as bleeding proceeds when the amounts of blood from the incisions fall. Indeed, thromboxane B<sub>2</sub> concentrations increased progressively, but the rate of production rose only during the first 3 min and thereafter remained constant at about 60 pg per min. This finding indicates that the growing plug of platelets produces increasing amounts of thromboxane A<sub>2</sub> only during an earlier stage of the haemostatic process. Since only a very small fraction of the total thromboxane-producing capacity of blood is utilised at sites of injury, only a small proportion of platelets at

risk presumably become involved at least as far as this component of haemostasis is concerned.

*Clotting venous blood in vitro* produced two orders of magnitude more thromboxane  $A_2$  than the same volume of bleeding-time blood and there was no correlation with bleeding time (IV). Determinations of the capacity of clotting blood to form thromboxane  $A_2$  *in vitro* does not therefore assist in evaluating the contribution of thromboxane  $A_2$  to the *in vivo* haemostatic situation.

If the haemostatic process in the skin incisions depended upon thromboxane  $A_2$  one might on the other hand expect that small variations in the amounts formed *in vivo* would be accompanied by corresponding variations in the bleeding time. Despite the correlation in appropriate groups of subjects between bleeding time and thromboxane  $B_2$  concentration in bleeding-time blood, in individuals the bleeding time was not correlated significantly with the production rate or the total production of thromboxane  $B_2$ . There was also no correlation between bleeding time and thromboxane  $B_2$  in the rising phase of bleeding in which the total amount of blood loss is related to the bleeding time. Therefore, the local formation of thromboxane  $A_2$  is not the only, or indeed the major determinant of skin bleeding time, which is the most reproducible parameter of haemostatic effectiveness *in vivo* in general use. Recent studies in human beings (Mac Nab et al, 1983) or in rats (Begent and Born, 1983) given thromboxane  $A_2$  – synthetase inhibitors also indicate that haemostasis depends not only or even to a major extent on thromboxane  $A_2$ .

It has been proposed that *fish in the diet* delays haemostasis by diminishing thromboxane  $A_2$  production by platelets (Dyerberg et al, 1978; Seiss et al, 1980; Dyerberg 1981; Leading article Lancet 1983).

When the volunteers were placed on the fish supplement diet and bleeding time was increased no changes occurred in the local appearance of *thromboxane  $A_2$  in vivo*, in the bleeding time blood, not even in those individuals whose bleeding times were increased most (V). Acetylsalicylic acid on the other hand greatly diminished the appearance of thromboxane  $A_2$  in the bleeding time blood in these same individuals and prevented the increase of thromboxane  $A_2$  concentrations with time (IV, V).

These findings confirm earlier conclusions (I, II, III) both that fish diets do not increase the bleeding time by diminishing the production of thromboxane  $A_2$  by platelets involved in the haemostatic process and that the delay in primary haemostasis ought to be due to a different mechanism than that of ASA.

Further support for these conclusions is that ASA and a dietary supplement of fish both of which increase the bleeding time significantly affected the *rate of blood loss* from the skin incisions differently (III). After ASA the rate of blood loss was significantly greater at all times as bleeding proceeds compared to controls. However, during the fish diet, when bleeding time was increased, there was no change in the rate of blood loss until the last 2–3 min during which bleeding



continued at a very low rate, as if the blood was leaking from only a few vessels in which haemostatic plugging was delayed.

In striking contrast to the absence of influence on *in vivo* local thromboxane  $A_2$  formation by the dietary fish, the production of thromboxane  $A_2$  in the same individuals *venous blood clotting in vitro* was significantly decreased already within one week on the diet when bleeding time was still unaffected, throughout the diet and even for 18 weeks afterwards, i.e. long after the normalization of the bleeding time (V). Thus the time course of this decrease was independent of the effect on *in vivo* bleeding time giving further support to the conclusion that the capacity of clotting blood to form thromboxane  $A_2$  appears to have no indicative value on the haemostatic situation (IV).

The decreased formation of thromboxane  $A_2$  *in vitro* agrees with similar decreases in human volunteers given a macherel diet for 7 days (Seiss et al, 1981) or receiving either cod liver oil or corn oil (Bronx et al, 1981). Similarly also, in those on cod liver oil the thromboxane  $A_2$  formed *in vitro* had not returned to control values four months after the end of the diet. Reduced *in vitro* formation of thromboxane  $A_2$  have thus been demonstrated in several investigations (Seiss et al, 1981; Dyerberg 1981; Bronx et al, 1981; Terano et al, 1983) in humans given fish oil supplements to the diet and therefore taken as evidence for the mechanism responsible for the effect of  $\omega$ -3 fatty acids on *in vivo* haemostasis.

One way to explain the discrepancy between the effect of the fish diet on thromboxane  $A_2$  formation *in vitro* and *in vivo* is through the assumption that in clotting venous blood all platelets are activated maximally and produce all the thromboxane  $A_2$  that can be formed from the arachidonic acid available for that conversion. *In vivo*, on the other hand only a small proportion of platelets "at risk" are activated and/or only parts of their total thromboxane  $A_2$  forming capacity are used so that in bleeding time blood much less thromboxane  $A_2$  is formed and no difference appears between diet and control. That platelets are activated maximally in clotting venous blood is suggested by the similarity in thromboxane  $A_2$  formation in these experiments and in others in which platelets were activated near maximally with collagen (Bronx et al, 1981). Partial replacement of convertible arachidonic acid by other fatty acids from the fish diet would then theoretically reduce the amount of thromboxane  $A_2$  that can be produced. This explanation can, however, only account for reduced thromboxane  $A_2$  formation while the fatty acid composition of platelet phospholipids is altered by the dietary fish, and not for the persistence in the reduction after the fatty acids have returned to normal. The persistence of the *in vitro* effects of the diet after its end could be explained by assuming that the diet alters some other, unknown factor or factors with biological time-constants sufficiently long for indirect expression through the parameters that were determined.

The proposed *effects of dietary fish* upon thrombosis and atherosclerosis may be mediated not only through its *influence on haemostasis* but also through an

*influence on lipids* and lipoprotein metabolism. It has been reported that dietary fish may lower triglycerides (TG), very low density lipoproteins (VLDL) and low density lipoproteins (LDL) and usually raise high density lipoproteins (HDL), all of which are considered desirable (Leading article Lancet 1983). Also from the present dietary investigations there is evidence for such effects (Thorngren et al, to be published).

## Concluding remarks

The observations of the present investigation (I–V) show that addition of fish to the diet causes primary haemostasis to be delayed similar to that caused by acetylsalicylic acid (ASA) but by a different mechanism. The findings indicate that dietary fish may have antithrombotic properties in man. The haemostatic effects caused by the dietary fish cannot, however, be accounted for by diminished local production of thromboxane  $A_2$  as commonly proposed, nor directly by decreased platelet aggregability or by the increase of dietary  $\omega$ -3 fatty acids in the platelets. The available data also indicate that thromboxane  $A_2$  is not the only or indeed the major determinant of the skin bleeding time. Since haemostasis depends not only on platelets and their capacity to produce thromboxane  $A_2$  but also on the reactivity of the injured vessels, red cells and on plasma coagulation mechanism, changes in these variables may be responsible partly or wholly for the delay in haemostasis associated with dietary fish. Recent studies in human beings and in rats given highly purified eicosapentaenoic acid also indicate effects on rheological properties (Terano et al, 1983) and on the reactivity of vessels by pressure hormones (Lorenz, et al, 1983; Begent et al, 1983). It has also been reported that dietary fish may lower triglycerides, VLDL and LDL and usually raise HDL, all of which are considered desirable (Leading article, Lancet 1983).

The understanding of a possible role of dietary fish and fish oils in thrombosis and atherosclerosis is at an early stage and much experimental and clinical work is needed before a full picture emerges. The observations of the present investigation, in conjunction with those of others are however, so far, compatible with the proposition that dietary fish, alone or in combination with drugs may diminish, by some mechanism or other, the incidence of arterial thrombosis in human populations.

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Paper I





## EFFECTS OF 11-WEEK INCREASE IN DIETARY EICOSAPENTAENOIC ACID ON BLEEDING TIME, LIPIDS, AND PLATELET AGGREGATION

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**Summary**      The effect of a diet rich in eicosapentaenoic acid (EPA) on platelet phospholipid fatty acid composition, platelet aggregation, and bleeding time was studied in 10 healthy men, whose usual diet was partly replaced by fish for 11 weeks. This diet provided 2-3 g EPA per day. Two doses (3.5 and 10 mg/kg body-weight) of acetylsalicylic acid (ASA) were given before and during the diet. The fish diet prolonged bleeding time (by 42%) and decreased platelet aggregability. The changes in platelet phospholipid fatty acid composition consisted of increases in the  $\omega$ -3 series (C20:5 and C22:6) and decreases in the  $\omega$ -6 series (C18:2 and C20:3). The reduction in platelet aggregation induced by collagen and ADP did not parallel the changes in platelet membrane phospholipids and bleeding times. Diminished platelet aggregation induced by collagen lasted only 3 weeks (while subject was still on the diet), whereas the decreased sensitivity to ADP persisted for at least 11 weeks after the volunteers had resumed their normal diet. ASA taken before the diet prolonged bleeding time by as much as did the diet itself. ASA taken during the diet prolonged bleeding time by more than the sum of the increases in bleeding time caused by ASA and by the EPA diet separately, but the synergism was not significantly more than additive. The findings suggest that a diet rich in  $\omega$ -3 polyunsaturated fatty acids reduces the interaction between platelets and the vessel wall by mechanisms which are more complex than just a reduction in susceptibility of platelets to the naturally occurring agents collagen and ADP, or an imbalance between proaggregatory and anti-aggregatory prostaglandin derivatives.

### Introduction

EPIDEMIOLOGICAL studies have shown that atherosclerotic cardiovascular disease is uncommon among Greenland Eskimos.<sup>1,2</sup> These Eskimos have lower plasma cholesterol and beta-lipoproteins, and much lower plasma triglycerides, than do mainland Danes.<sup>3</sup> These differences are due to

differences in diets. The Eskimos also have high plasma levels of eicosapentaenoic acid (EPA), the source of which is the diet.<sup>4-6</sup>

These observations have led to a hypothesis which proposes that large quantities of  $\omega$ -3 polyunsaturated fatty acids, mainly EPA, in the diet protects Eskimos against the thromboembolic complications of cardiovascular diseases by reducing platelet aggregability.<sup>6</sup>

Various mechanisms have been proposed to account for the decreased platelet aggregability of people on such diets. It has been suggested that EPA can be used by vessel walls to make prostaglandin I<sub>3</sub>, which is as potent as prostaglandin I<sub>2</sub> (prostacyclin) in inhibiting platelets; whereas the platelets themselves would produce thromboxane A<sub>3</sub> which, unlike thromboxane A<sub>2</sub>, has no aggregating effect.<sup>6</sup> The anti-thrombotic condition brought about by such diets would then be due to an imbalance between opposing prostaglandin derivatives, with the platelet inhibitory effect predominant.

According to the same hypothesis, long-term dietary enrichment with EPA should prolong bleeding time because of reduced platelet aggregability which, in turn, should be related to changes in the fatty acid composition of platelets and plasma lipids.

To elucidate further the possible connections between diet and haemostasis which could explain the low incidence of thrombotic disease in Eskimos, a study was conducted in Lund during the winter of 1980-81. This investigation differs from previous ones, which either consisted of epidemiological surveys of Greenland Eskimos<sup>2,7</sup> or studied the effects of short-term dietary changes in volunteers.<sup>8</sup>

## Methods

### *Subjects*

The subjects were ten apparently healthy male volunteers, non-smokers, who were students in Lund and were aged 28-35 years. They had not taken drugs in the 2 months before the study, nor did they take any during the investigation. For the study they took their usual Swedish diet, the only changes being abstinence from alcohol for the 3 days before blood samples were taken and a predominance of fish, mainly mackerel and salmon, in the diet for 11 weeks. This diet was well tolerated. It increased EPA (C20:5,  $\omega$ -3) intake by 2-3 g per day.

Blood samples were taken at the following times: on two occasions 2 weeks apart during the baseline, pre-diet period (values shown in the figures are those of the first sample); after 3, 6, and 11 weeks of the diet; and after 6 and 11 weeks in the post-diet period. Samples were taken in the morning after an overnight fast of 12 h. In the

baseline period the first sampling was done from both left and right arms in order to check whether bleeding time, platelet aggregation, and lipid composition would be affected by technical variability. There were no significant differences between measurements of samples from the two arms except for platelet aggregation, for which maximum optical density change induced by collagen (4  $\mu\text{g/ml}$ ) was 18% higher for samples from the right arm than those for the left.

### *Lipid Analyses*

Platelets were prepared for lipid analyses according to a method already described,<sup>9</sup> with minor modifications. Lipids were extracted with chloroform-methanol 2:1 in the presence of butylated hydroxytoluene. Platelet phospholipids were separated by thin-layer chromatography on 0.5 mm silica gel with chloroform-methanol-acetic acid-water (65:25:4:4 v/v). The separated fraction containing phosphatidylcholine (PC), phosphatidylinositol, and phosphatidylserine was methylated and analysed for fatty acids by gas-liquid chromatography on a 'Varian 3700' equipped with a flame-ionisation detector on a 200 cm glass column (2 mm internal diameter) packed with 15% diethyleneglycol succinate (DEGS) and coated with 'Chromosorb W', 80–100 mesh. The column was operated at 190°C with nitrogen as carrier gas. Individual esters were identified by comparison with standards of phospholipid fatty acid methylesters. Peaks were measured and the fatty acid distribution was calculated by a computer CDS 111 (17:0 heptadecanoic acid was used as internal standard).

### *Tests of Haemostasis*

Bleeding times<sup>10</sup> were determined by the standardised 'Simplate II' device from General Diagnostics.<sup>11</sup> Platelets were counted in 'Ultra Flow 100' (Clay Adams). Fibrinogen levels,<sup>12</sup> activated partial thromboplastin time,<sup>13</sup> and prothrombin time (by the use of a LODE-coagulometer) were determined.

### *Platelet Aggregation*

Venous blood from fasting subjects was centrifuged at 275 *g* at room temperature for 10 min to produce platelet-rich plasma. The red cell fraction was re-centrifuged at 2600 *g* for 20 min to produce platelet-poor plasma. Platelet aggregation was measured photometrically<sup>14</sup> in a Payton aggregometer. The aggregating agents were collagen (Hormon-Chemie, Munich), 0.5, 1.0, 2.0, and 4.0  $\mu\text{g/ml}$ ; adenosine diphosphate (Sigma, St Louis, U.S.A.); and adrenaline (adrenaline hydrogen-tartrate, B.D.H. Biochemicals, U.K.).

The indices of aggregation were: (1) maximum decrease in optical density expressed as a percentage of the difference in optical density between platelet-rich and platelet-poor plasma, and (2) the lowest concentration of ADP or adrenaline which induced secondary aggregation, i.e., the release reaction. This concentration is referred to as the threshold value of ADP or adrenaline.

### *Effect of Acetylsalicylic Acid (ASA)*

Bleeding times were determined before and 2 h after the oral administration of ASA 3.5 mg/kg and 10 mg/kg body-weight during the baseline period (the effects of the two doses were determined two weeks apart) and after 11 weeks of the diet (when the higher dose was given 2 h after the lower dose).

### *Statistical Evaluation*

Student's *t* test was used for lipid analyses and Wilcoxon's test for those of bleeding times and platelet aggregation.

## **Results**

### *Changes in Platelet Lipids During the Diet*

The EPA-rich diet produced changes in the fatty acid composition of the phosphatidylcholine (lecithin) of the platelets (see accompanying table). After 3 weeks the proportion of C20:5 and C22:6 ( $\omega$ -3) polyunsaturated fatty acids increased ( $p < 0.001$ ); and so did the C22:5 ( $\omega$ -3) acids ( $p < 0.001$ ) after 6 weeks. These changes persisted until the end of the dietary period.

The proportion of the more saturated ( $\omega$ -6) fatty acids fell after 3 weeks—C18:2 ( $p < 0.05$ ) and C20:3 ( $p < 0.05$ ). The proportion of arachidonic acid, C20:4, remained practically unchanged throughout. The three-fold increase in fatty acid ratio C20:5/C20:4 was due mainly to the increase in proportion of C20:5. At 6 and 11 weeks after the end of the dietary period the fatty acid composition of the platelet lecithin had reverted to the pre-dietary pattern.

### *Bleeding Time*

Bleeding time increased by an average of 31% ( $p < 0.01$ ) 3 weeks after the start of the diet; the maximum increase (42%,  $p < 0.01$ ) was reached after 6 weeks; and after 11 weeks the bleeding time was still prolonged (by 33%,  $p < 0.05$ ) (fig. 1). Bleeding times measured in the post-dietary period had reverted to baseline values.

Before, during, and after the dietary period, there were no significant changes in blood platelet concentration; fibrinogen concentration; prothrombin time; and activated partial thromboplastin.

### *Platelet Aggregation*

Aggregation induced by collagen was depressed after 3 weeks of the fish diet, whether the dose of collagen used was

FATTY ACID COMPOSITION (%) INCLUDING SUM OF  $\omega$ -3 AND  $\omega$ -6 FATTY ACIDS IN PLATELET PHOSPHATIDYL CHOLINE INDUCED BY AN EPA-ENRICHED (2-3 g/day) DIET

|                            | 16:0           | 18:0           | 18:1          | 18:2          | 20:3          | 20:4           | 20:5          | 22:5          | 22:6          | $\omega$ -3   | $\omega$ -6    |
|----------------------------|----------------|----------------|---------------|---------------|---------------|----------------|---------------|---------------|---------------|---------------|----------------|
| <i>Baseline</i>            |                |                |               |               |               |                |               |               |               |               |                |
| (0 weeks)                  | 21.3<br>(0.3)  | 22.0<br>(0.4)  | 23.7<br>(0.2) | 7.1<br>(0.2)  | 1.8<br>(0.1)  | 18.3<br>(0.6)  | 0.4<br>(0.1)  | 0.9<br>(0.1)  | 1.7<br>(0.1)  | 4.3<br>(0.2)  | 28.6<br>(0.5)  |
| <i>Dietary period</i>      |                |                |               |               |               |                |               |               |               |               |                |
| 3 weeks                    | 22.5†<br>(0.4) | 21.4<br>(0.2)  | 23.8<br>(0.3) | 6.1*<br>(0.2) | 1.5*<br>(0.1) | 17.5<br>(0.4)  | 1.2#<br>(0.1) | 1.0<br>(0.1)  | 2.5#<br>(0.1) | 5.9#<br>(0.3) | 26.3†<br>(0.6) |
| 6 weeks                    | 22.3*<br>(0.4) | 21.5<br>(0.4)  | 23.9<br>(0.3) | 6.0*<br>(0.3) | 1.4*<br>(0.1) | 16.8<br>(0.5)  | 1.4#<br>(0.2) | 1.1#<br>(0.1) | 2.5#<br>(0.1) | 6.6#<br>(0.3) | 25.5#<br>(0.7) |
| 11 weeks                   | 22.6#<br>(0.3) | 20.9†<br>(0.2) | 23.7<br>(0.5) | 5.9*<br>(0.3) | 1.4*<br>(0.1) | 16.9<br>(0.4)  | 1.6#<br>(0.2) | 1.2#<br>(0.1) | 2.7#<br>(0.2) | 7.0#<br>(0.3) | 25.8#<br>(0.6) |
| <i>Post dietary period</i> |                |                |               |               |               |                |               |               |               |               |                |
| 6 weeks                    | 22.3<br>(0.6)  | 21.7<br>(0.2)  | 23.6<br>(0.2) | 6.7<br>(0.3)  | 1.7<br>(0.1)  | 17.9<br>(0.6)  | 0.5<br>(0.1)  | 0.8<br>(0.1)  | 1.7<br>(0.1)  | 4.5<br>(0.2)  | 28.0<br>(0.5)  |
| 11 weeks                   | 20.3†<br>(0.3) | 21.40<br>(0.3) | 24.3<br>(0.3) | 6.5<br>(0.2)  | 1.8<br>(0.1)  | 20.0*<br>(0.4) | 0.5<br>(0.1)  | 1.0*<br>(0.1) | 1.8<br>(0.1)  | 4.3<br>(0.2)  | 29.8<br>(0.4)  |

Values given are mean; SEM in parentheses. \*  $p < 0.05$ . †  $p < 0.01$ . #  $p < 0.001$ .



Platelet lipid composition

|                        |      |      |      |      |      |      |
|------------------------|------|------|------|------|------|------|
| C 20:5                 | 0.4  | 1.2  | 1.4  | 1.6  | 0.5  | 0.2  |
| C 20:4                 | 18.3 | 17.5 | 16.8 | 16.9 | 17.9 | 18.4 |
| Ratio $\times 10^{-3}$ | 22   | 69   | 83   | 95   | 28   | 11   |

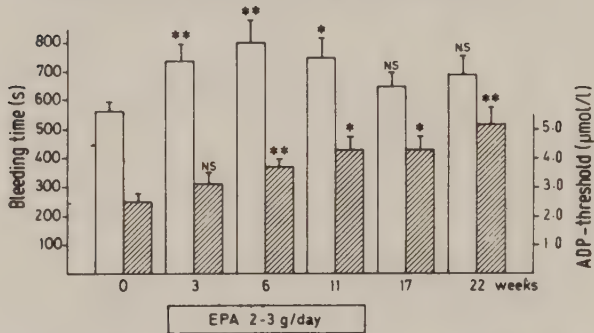


Fig. 1—Effect of EPA-rich diet on in-vivo bleeding time (open column); platelet sensitivity to ADP in vitro (hatched column), recorded as “ADP-threshold”; and platelet lipid composition.

Values given are mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with control values at 0 weeks.

the lowest which induced aggregation at all ( $p < 0.01$ ), or 1  $\mu\text{g/ml}$  ( $p < 0.05$ ), 2 ( $p < 0.05$ ), or 4 ( $p < 0.01$ ) (fig. 2).

The smallest concentration of ADP required to induce the second phase of aggregation, i.e., the ADP-threshold, was raised during the dietary period (fig. 1). This evidence of decrease in platelet aggregability was observed 6 weeks after the start of the diet ( $p < 0.01$ ) and lasted until after the dietary

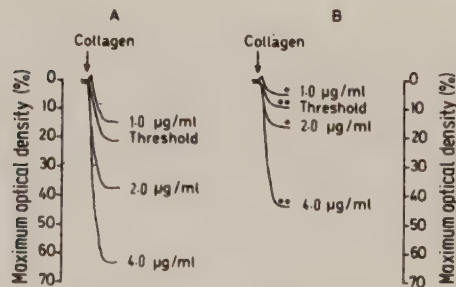


Fig. 2—Platelet aggregation (mean maximum optical density) by collagen before (A) and after (B) 3 weeks of EPA-enriched diet.

\* $p < 0.05$ , \*\* $p < 0.01$ .

period; the significance of the decrease was ( $p < 0.05$ ) at 11 weeks of the dietary period, and  $p < 0.05$  and  $p < 0.01$  at 6 and 11 weeks, respectively, after the end of the diet.

Platelet aggregation induced by adrenaline did not change significantly during the dietary period.

#### *Effects of Acetylsalicylic Acid (ASA) on Bleeding Time*

During the baseline period 3.5 mg/kg ASA prolonged bleeding time by 29% ( $p < 0.05$ ), and 10 mg/kg prolonged it by 41% ( $p < 0.01$ ) (fig. 3). At the end of the dietary period ASA prolonged bleeding time by 55% ( $p < 0.01$ ) and 44% ( $p < 0.05$ ) after doses of 3.5 and 10 mg/kg body-weight, respectively (figs 3 and 4).

#### Discussion

The purpose of this investigation was to maintain healthy young men for several weeks on an ordinary Swedish diet that was partially replaced by an acceptable amount of fish and to relate the expected haemostatic abnormalities to changes in platelet lipids. The diet was well tolerated by the volunteers.

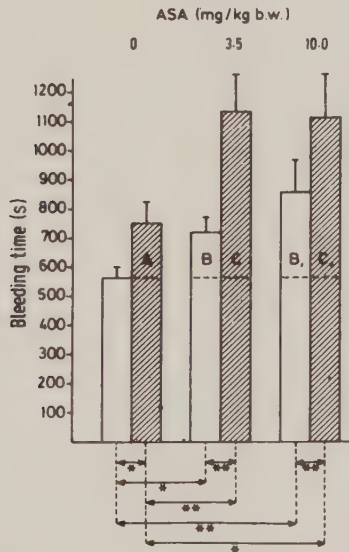


Fig. 3—Effect of acetylsalicylic acid (ASA) on bleeding time before (open column) and during 11th week of (hatched column) EPA-rich diet.

\* $p < 0.05$ , \*\* $p < 0.01$ .

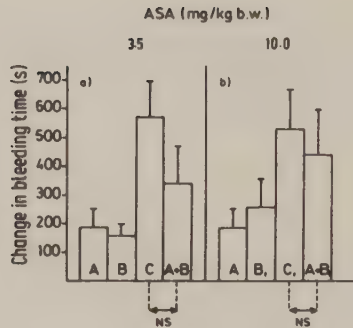


Fig. 4—Increase in bleeding time (mean±SEM) produced by EPA-rich diet at 11 weeks (A); ASA, 3.5 mg/kg body-weight (B) or 10 mg/kg body-weight (B1); and EPA-rich diet plus ASA 3.5 mg/kg body-weight (C) or 10 mg/kg body-weight.

It provided an intake of 2–3 g EPA per day; this was considerably more than would have been consumed otherwise, but less than the average taken by Greenland Eskimos.<sup>5</sup>

Three weeks after the start of the diet the expected changes in fatty acid composition of platelet membrane phospholipids occurred—there were increases in polyunsaturated fatty acids belonging to the  $\omega$ -3 series (i.e., C20:5 and C22:6) and decreases in the fatty acids of the  $\omega$ -6 series (i.e., linoleic acid C18:2 and D-homo-linoleic acid C20:3). The three-fold increase in ratio of C20:5 to C20:4 (i.e., EPA to arachidonic acid) was due mainly to an increase in EPA; however, this ratio (0.08) was not nearly as great as that which occurred in Greenland Eskimos (0.94).<sup>7</sup> The reduction in linoleic acid (C18:2) content could not be explained by a deficiency of this or other essential fatty acids in the diet.

Within 3 weeks the fish diet produced prolonged bleeding time and reduced platelet aggregability. Bleeding times remained prolonged for the rest of the dietary period, but had returned to baseline values when measured 6 and 11 weeks after the end of the diet, as did the proportion of  $\omega$ -3 fatty acids in the platelet phospholipids. These results support the suggestion of a causal relation between the proportion of the  $\omega$ -3 fatty acids in platelet membrane phospholipids and in bleeding time.

The aggregability of the platelets dropped 3 weeks after the start of the fish diet, whether aggregation was induced by collagen or by ADP. This observation was in accordance with

the low aggregability of platelets from Greenland Eskimos, who live largely on fish. However, aggregation by collagen returned to pre-dietary values 6 and 11 weeks after the start of the diet; and the decrease in sensitivity to ADP, defined as the smallest ADP concentration required to induce secondary aggregation, persisted until at least 11 weeks after the end of the dietary period. Therefore, the effects of the EPA-rich fish diet on platelet membrane phospholipids and on the in-vivo bleeding time did not parallel its effect on in-vitro platelet aggregation, with a further divergence according to whether aggregation was induced by collagen or by ADP.

ASA is thought to inhibit secondary aggregation and the release reaction by acetylating the cyclo-oxygenase of platelets and thus preventing them from synthesising the aggregating agent thromboxane  $A_2$ .<sup>15,16</sup> ASA given before the diet prolonged bleeding time by as much as did the diet alone (fig. 3). ASA given during the diet prolonged bleeding time by somewhat more than the sum of the increases caused by ASA and by the EPA diet separately, but the synergism was not significantly more than additive (fig. 4). This suggests that the EPA diet does not reduce the interaction between platelets and vessel walls exclusively by altering prostaglandin synthesis (i.e., by reducing activity of cyclo-oxygenase).

Our results suggest that  $\omega$ -3 polyunsaturated fatty acid supplements prolong bleeding time by mechanisms which are more complex than the decrease in susceptibility of platelets to the naturally occurring agents collagen and ADP, or by causing an imbalance between pro-aggregatory and anti-aggregatory prostaglandin derivatives.

There are good reasons for doubting whether such an imbalance is really likely to account for the prolongation of bleeding time. Thromboxane  $A_2$  is a very potent platelet aggregating agent and it is produced in vitro in large quantities by aggregating platelets. If this occurs also in vivo, it seems improbable that a fairly small, say 20%, decrease in thromboxane  $A_2$  production would diminish haemostatic platelet aggregation sufficiently to produce the observed increases in bleeding time. The possibility then remains that thromboxane  $A_3$  impairs the aggregating effect of thromboxane  $A_2$  and that it is produced in sufficient amounts to prolong bleeding time.

There is strong evidence that a major proportion of acute coronary thromboses are initiated by haemorrhage through fissures in atheromatous plaques.<sup>17-19</sup> Since the thrombi begin as aggregates of platelets the mechanism initiating coronary thrombosis is likely to be similar to that responsible

for the haemostatic aggregation of platelets following traumatic injury to vessels elsewhere. However, since bleeding time is affected not only by platelet aggregation but also by plasma coagulation factors, by the red blood cells, and by vascular contractility, changes in these variables may be responsible partly or wholly for the prolongation of bleeding times associated with a fish diet.

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Paper II



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## Effects of Acetylsalicylic Acid on Platelet Aggregation before and during Increase in Dietary Eicosapentaenoic Acid

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**Key Words.** Acetylsalicylic acid · Eicosapentaenoic acid · Platelet aggregation

**Abstract.** The effect of acetylsalicylic acid (ASA) on platelet aggregation before and during a fish diet, already known to decrease the aggregability of platelets and to prolong the bleeding time, was studied in 10 healthy men. Two doses (3.5 and 10 mg/kg body weight) of ASA were given. Both doses equally decreased platelet aggregation to collagen and adenosine diphosphate (ADP). ASA, taken before the diet, diminished platelet aggregability to ADP by as much as did the diet alone. When ASA was administered during the diet, the effect on platelet aggregability to ADP was additive. Aggregation to collagen also decreased to the same extent as during the baseline period. The results, in conjunction with our earlier ones, indicate that the mechanism by which a fish diet delays primary haemostasis is different from the apparently similar effect of ASA. This raises the possibility of augmenting any antithrombotic effect of ASA by dietary means.

### Introduction

Marine fatty acids, mainly eicosapentaenoic acid (EPA), has been linked to the low incidence of atherosclerotic cardiovascular disease in Greenland Eskimos [1]. It has been suggested that EPA diminishes the interaction between platelets and vessel walls by causing an imbalance between pro- and

antiaggregatory prostaglandin derivatives formed by the platelets and the vessel wall, respectively [2].

We recently reported prolongation of bleeding time and reduction of platelet aggregability in healthy volunteers, whose normal Swedish diet was partly replaced by fish [3]. The effect on bleeding time did not parallel that on in vitro platelet aggregation. Acetyl-



salicylic acid (ASA), in doses which inhibit prostaglandin synthesis almost completely, prolonged the bleeding time by as much as the diet did, and when given during the diet its effect was additive. This evidence provided support for doubting that a simple imbalance between proaggregatory and antiaggregatory prostaglandins could account for the prolongation of bleeding time. This doubt is augmented by this report on the comparative effects of ASA, given before and during the same fish diet, on platelet aggregation.

## Methods

### *Subjects*

The volunteers were 10 apparently healthy men, non-smokers, and aged 28–35 years. They had not taken drugs in the 2 months before the study, nor did they take any during the investigation. During the dietary period they took their normal Swedish diet, the only changes being abstinence from alcohol for the 3 days before blood samples were taken and a predominance of fatty fish, in the diet for 11 weeks. The diet increased EPA (C20:5,  $\omega$ -3) intake by 2–3 g per day.

### *Effect of Acetylsalicylic Acid*

Platelet aggregation was determined before and 2 h after the oral administration of ASA, 3.5 and 10 mg/kg body weight, during the pre-dietary period (the effects of the two doses were determined 2 weeks apart) and after 11 weeks on the diet (when the higher dose was given 2 h after the lower dose).

### *Platelet Aggregation*

Venous blood was centrifuged at 275 *g* at room temperature for 10 min to produce platelet-rich plasma. Platelet-poor plasma was prepared by re-centrifuging the red cell fraction at 2,600 *g* for 20 min. Platelet aggregation was measured photometrically [4] in a Payton aggregometer. The aggregating agents were collagen (Hormon-Chemie, Munich), 1.0, 2.0, 4.0  $\mu$ g/ml, and adenosine diphosphate (ADP; Sigma, St. Louis, Mo.)

The indices of aggregation were for collagen: (1) the maximum decrease in optical density, expressed as a percentage of the difference in optical density, ex-

pressed as a percentage of the difference in optical density between platelet-rich and platelet-poor plasma, and (2) the lowest concentration of ADP which induced secondary aggregation, i.e., the release reaction. This concentration is referred to as the threshold value of ADP.

Values given are mean value  $\pm$  standard error of the mean.

Statistical evaluation of the results was done by Student's *t* test.

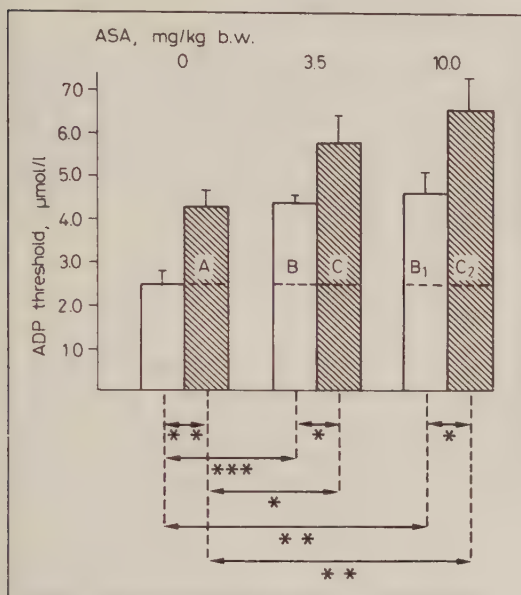
## Results

### *ADP*

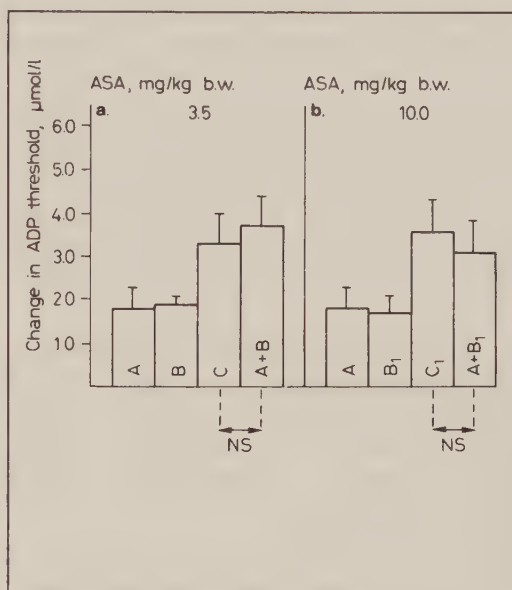
The smallest concentration of ADP required to induce the second phase of aggregation, i.e. the ADP threshold, was  $2.5 \pm 0.3 \mu$ mol during the baseline period and raised after 11 weeks on the diet to  $4.3 \pm 0.5 \mu$ mol (fig. 1). During the baseline period, 3.5 mg ASA/kg b.w. increased the ADP threshold to  $4.4 \pm 0.2 \mu$ mol ( $p < 0.001$ ), and 10 mg/kg b.w. to  $4.6 \pm 0.5 \mu$ mol ( $p < 0.01$ ) (fig. 1, 2). At the end of the dietary period, ASA increased the ADP threshold to  $5.8 \pm 0.7$  and  $6.7 \pm 0.7 \mu$ mol after 3.5 and 10 mg/kg b.w., respectively (fig. 1, 2).

### *Collagen*

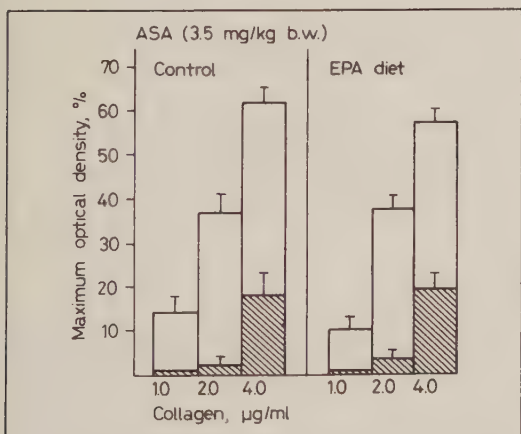
During the baseline period, 3.5 mg ASA/kg b.w. decreased platelet aggregation by collagen ( $p < 0.01$ ) (fig. 3). There was no further decrease after 10 mg ASA/kg b.w. The diminished platelet aggregation to collagen induced by the diet lasted only 3 weeks and increased to pre-dietary values while the volunteers were still on the diet [3]. At the end of the dietary period, 3.5 mg ASA/kg b.w. again decreased platelet aggregation to the same extent as during the baseline period. There was no further decrease after 10 mg ASA/kg b.w.



**Fig. 1.** Effect of ASA on platelet aggregability by ADP, measured as ADP threshold, before (open column) and during the 11th week (hatched column) of EPA-rich diet. Values given are mean  $\pm$  SEM. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .



**Fig. 2.** Increase in the ADP threshold (mean  $\pm$  SEM) produced by EPA-rich diet at 11 weeks (A); ASA, 3.5 mg/kg b.w. (B) or 10 mg/kg b.w. (B<sub>1</sub>); and EPA-rich diet plus ASA, 3.5 mg/kg b.w. (C) or 10 mg/kg b.w. (C<sub>1</sub>). Values given are mean  $\pm$  SEM. NS = Not significant.



**Fig. 3.** Effect of ASA on platelet aggregation (mean maximum optical density) by collagen before and during the 11th week of EPA-rich diet. Values given are mean  $\pm$  SEM.

## Discussion

The purpose of the present investigation was to evaluate and relate the decrease in platelet aggregability by ASA to that caused by a fish diet rich in EPA.

ASA, in doses which inhibit prostaglandin synthesis almost completely, given before the fish diet decreased platelet aggregability to ADP by as much as did the diet alone. The decrease in platelet aggregability was similar, irrespective of the dose of ASA given (3.5 or 10 mg/kg b.w.). When ASA was administered during the diet, the effect on platelet aggregability by ADP was additive. This effect is in accordance with our earlier observations on a similar additive bleeding time pro-

longation by ASA during the fish diet [3]. These findings provide support for doubting that marine fatty acids reduce the interaction between platelets and vessel walls exclusively by altering prostaglandin synthesis, i.e., by reducing the activity of cyclo-oxygenase. Furthermore, when ASA was given during the diet, aggregation to collagen also decreased to the same extent as during the baseline period.

We have already reported that a fish diet, which increases the bleeding time and changes the fatty acid composition of platelets, decreases their aggregability to both ADP and collagen [3]. Particularly interesting are the observations that the reactivity to collagen became normal again well before the end of the diet, whereas the diminished reactivity to ADP persisted long after the end of the diet and the normalization of the bleeding time. There is, therefore, no correlation between these parameters, which makes a causative role of solely the platelet changes in the prolonged bleeding time unlikely.

The results, in conjunction with the earlier ones [3], indicate that the mechanism by which a fish diet delays primary haemostasis is different from the apparently similar effect of ASA on platelet aggregation and bleeding time prolongation. This raises the possibility of augmenting any antithrombotic effect of ASA by dietary means.

## Acknowledgements

This study was supported by the Faculty of Medicine, University of Lund, and was sponsored by the Swedish Medical Research Council (project B 82-19X-05701), Swedish Margarine Industries' Association for Nutritional Research, and Albert and Gerda Svensson's Foundation.

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Paper III



QUANTIFICATION OF BLOOD FROM SKIN BLEEDING TIME  
DETERMINATIONS:  
EFFECTS OF FISH DIET OR ACETYLSALICYLIC ACID

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## ABSTRACT

The Simplate II technique for measuring bleeding time was adapted to quantify the volume of blood as a function of time and for determining how this parameter was affected by either acetylsalicylic acid (ASA) or a dietary supplement of fish. Both increased the bleeding time significantly. Irrespective of the final bleeding time, the rate of blood loss increased for about the first 2 min and then decreased almost linearly until bleeding stopped. This time course was not affected by either ASA or the fish diet. The lack of effect by ASA suggests that the initial increase in bleeding time, presumably due to reversal of vasoconstriction, does not involve prostaglandin derivatives.

After ASA the rate of blood loss was significantly greater throughout, probably caused by deceleration of platelet aggregation. During the fish diet, when bleeding time was increased, there was no change in the rate of blood loss until the last 2-3 min during which bleeding continued at a very low rate. The results support the conclusion that the delay in primary haemostasis produced by a fish diet is due to a different mechanism than that produced by ASA.

## INTRODUCTION

The effectiveness of primary haemostasis is usually quantified by determining a bleeding time (1,2), i.e. the time from a standardised injury to the complete stopping of bleeding from it. This time is, however, only one out of several possible parameters of haemostatic effectiveness. Another parameter is the quantity of blood which emerges from a standardised injury (3, 4).

This paper reports a simple addition to the Simplate II technique (5, 6) for determining the bleeding time on standardised skin incisions. The addition is to quantify the blood emerging from the incisions, so that the bleeding time can be correlated with the time course and the total volume of blood loss. The effects on all three parameters of either a diet supplemented with fatty fish or acetylsalicylic acid (ASA) were determined, both of which are known to prolong the bleeding time (7, 8) and are under investigation for the prevention of thromboembolic complications of cardiovascular disease (7, 9-11).

## METHODS

### Subjects

The subjects were nineteen apparently healthy Swedish male volunteers, aged 21-39 ( $29 \pm 1,0$ , mean  $\pm$  SEM) all non-smokers. They had taken no drugs for at least two weeks before the study nor did they take any during the investigation. Bleeding times and the bleeding time blood volumes were determined before and two hours after the oral administration of ASA, 5 mg/kg bodyweight.

Twelve of the volunteers were then put on a fish-rich diet. For the study they took their usual Swedish diet the only changes being abstinent from alcohol for three days before blood samples were taken and a predominance of fish mainly mackerel, salmon and herring in the diet for six weeks.

This diet was well tolerated. It increased the EPA (C20:5  $\omega$ -3) intake by 2-3 g per day. Bleeding times and bleeding time blood volumes were determined on two occasions two weeks apart during the baseline, pre-diet period (values given are mean of these two results), after 1, 3 and twice after 6 weeks (values given are mean of these two results) of the diet.

#### Measurements of haemostatic effectiveness

Bleeding times. Bleeding times were determined with the standard Simplate II device from General Diagnostics (5). The skin incision was blotted every 30 s with a disc of Whatman No. 1 filter paper.

Bleeding time blood volumes. Bleeding time blood volumes were determined by immersing each bleeding time blood spot on the filter paper in 6 ml of Drabkins solution. Spots containing more than 50  $\mu$ l were cut in two. To establish completeness of elution of blood from the filter paper by this procedure, known volumes of blood were spotted on filter paper, eluted, and the haemoglobin concentration was compared with that of the same volumes added directly to Drabkins solution. The correlation coefficient between these two procedures was highly significant ( $r=0.99$ ,  $n=40$ ) (Fig. 1). All blood was eluted from the filter paper after 2 h. In the eluate haemoglobin concentration was estimated spectrophotometrically (12) with a Vitatron Universal Photometer. The volume of the bleeding time blood was calculated from a standard curve: known volumes of peripheral blood taken at the same time from each subject were added to Drabkins solution and the haemoglobin concentrations were determined. Results were expressed as mikrolitre blood emerging in each 0.5 min period.

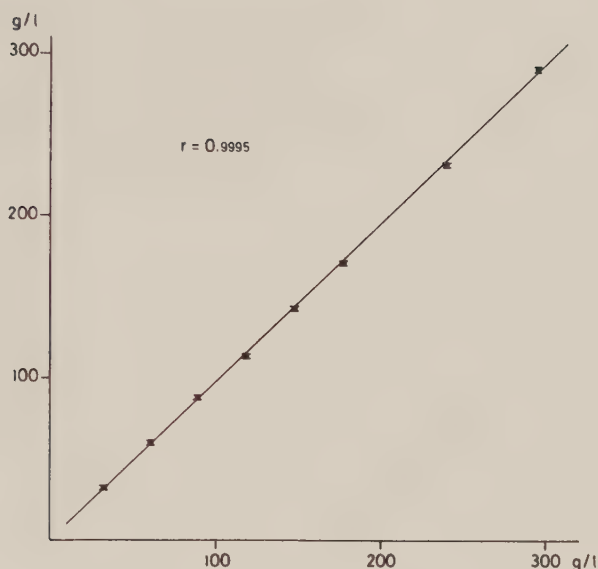


Fig. 1. Correlation between haemoglobin concentrations of known volumes of blood eluted from filter paper (abscissa) and the same volumes added directly to Drabkins solution (ordinate).

### Statistical Evaluation

Bleeding times and blood volumes were compared by Wilcoxon's test. Correlation coefficients were determined by regression analysis.

### RESULTS

In the nineteen young Swedish men the Simplate bleeding time was  $8.2 \pm 0.5$  min (mean  $\pm$  SEM). The total bleeding time blood volume was  $505 \pm 75$   $\mu$ l (mean  $\pm$  SEM). These total blood volumes were significantly correlated with the corresponding bleeding times ( $r=0.77$ ;  $p < 0.001$ ).

The volume of blood emerging from the cut increased in the first 2 min irrespective of the final bleeding time,

and then decreased continuously until bleeding ceased (Fig. 2). During control periods, i.e. before either fish or ASA, the total volumes of blood emerging from the time of incision up to the maximum were correlated significantly with the final bleeding times ( $r=0.66$ ;  $p < 0.01$ ).

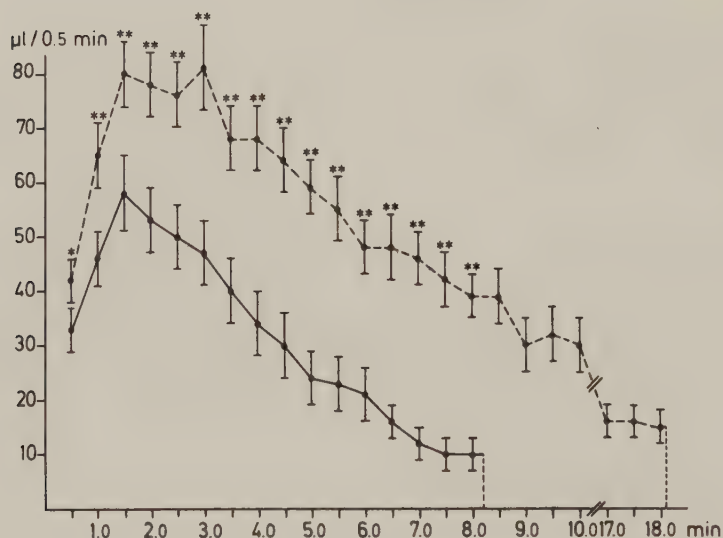


Fig. 2. Blood loss ( $\mu\text{l}/0.5 \text{ min}$ ) from skin bleeding time determinations (on 19 subjects) as a function of time (min) under control conditions (●—●) and after administration of ASA (5 mg/kg bodyweight) (●---●). Vertical bars indicate SEM; significance of differences  $p < 0.01$  (\*\*).

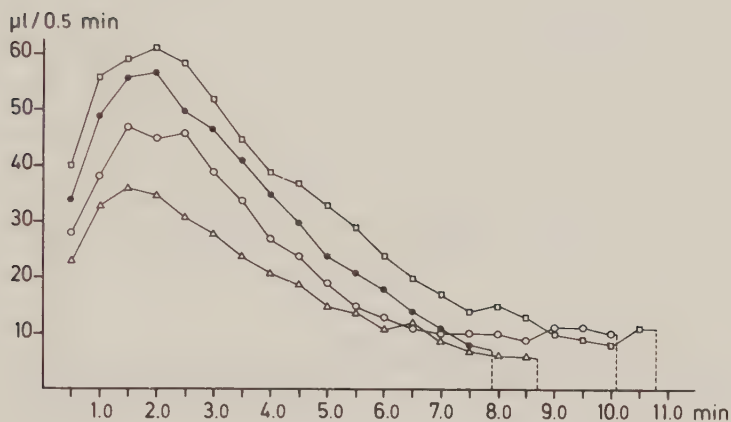


Fig. 3. Effects of fish supplement diet on blood loss ( $\mu\text{l}/0.5 \text{ min}$ ) from skin bleeding time determinations as a function of time (min): control (●—●) and after 1 (○—○), 3 (△—△) and 6 weeks (□—□) on the diet.

When twelve of the volunteers were placed on the fish supplement diet, the bleeding time did not increase significantly until the sixth week ( $p < 0.01$ , after 6 weeks). The bleeding time blood loss again increased to a maximum after about 2 min and then decreased progressively until 2-3 min before bleeding ceased; during that final period blood continued to emerge in small quantities (Fig. 3 and Fig. 4). The correlation between bleeding time and blood loss up to the 2 min maximum persisted, although less significantly ( $r=0.48$ ;  $p < 0.05$ ). After three weeks on the diet the bleeding time blood volumes/0.5 min were significantly less than before the diet ( $p < 0.01$ ) whereas after six weeks the volumes were not significantly different from controls.



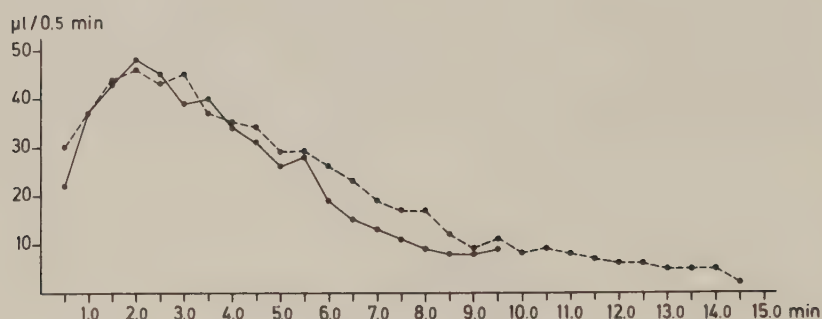


Fig. 4. Blood loss ( $\mu\text{l}/0.5 \text{ min}$ ) from skin bleeding time determinations as a function of time (min) for one of the volunteers during control condition (●—●) and after 6 weeks (●---●) on the fish diet.

The correlation between bleeding time and total blood loss persisted throughout the diet ( $r=0.73$   $p < 0.01$ ;  $r=0.63$   $p < 0.05$ ;  $r=0.66$   $p < 0.05$  after 1, 3 and 6 weeks respectively).

All nineteen volunteers took one dose of ASA of 5 mg/kg bodyweight. Their bleeding times increased to  $18.2 \pm 1.9$  min, i.e. by 134% (see Fig. 2). The total blood loss increased to  $1400 \pm 135$  l, i.e. by  $234 \pm 39\%$ . The blood loss versus time curve after aspirin was similar to that before, but the loss was very significantly greater at all times (Fig. 2). The correlation between bleeding time and total blood loss persisted ( $r=0.67$ ;  $p < 0.001$ ) but there was no longer a correlation between the bleeding time and the blood loss up to its maximum ( $r=-0.16$ ).

## DISCUSSION

Determination of the skin bleeding time by the Simplate technique is the most reproducible parameter of haemostatic effectiveness currently in clinical use. Like other bleeding

times, it is a measure of the duration of blood loss from the moment of injury to the complete cessation of bleeding. The technique provides no information about the rate and quantity of blood loss, nor how these may be affected by different physiological or pathological conditions or by pharmacological agents.

As part of our attempt to find out by what mechanism(s) the dietary intake of fatty fish increases the bleeding time (7), we have determined the quantity of blood emerging from skin incisions as a function of time and how this quantity is affected by a dietary supplement of fish or by acetylsalicylic acid (ASA), both of which increase the duration of bleeding from such incisions (7, 8). The results show that, irrespective of the final bleeding time, blood loss invariably increased for about the first two minutes. The duration of this increase was not affected by either the fish diet or ASA, suggesting that the initial bleeding is not influenced by prostaglandins which are affected by the diet (13, 14) or the drug (15, 16). The increase is explained most simply on the assumption that the incision rapidly caused local vasoconstriction (17) which disappeared in the subsequent 2 minutes or so.

After its initial increase the rate of blood loss decreased continuously and almost linearly until bleeding ceased. During the fish diet this decrease occurred similarly until the last two or three min during which bleeding continued at a very low rate, as if the blood were leaking from only a few vessels in which haemostatic plugging was delayed. After three weeks on the fish diet, when the bleeding time was still unaffected, the rate of blood loss was significantly decreased, although platelet aggregation by ADP in vitro was diminished from the first week on the diet (data not yet published).

After ASA the time course of the increase and decrease in bleeding was again similar to the control. However, the quantity of blood lost was significantly greater throughout. A probable although not the only explanation for this is the slowing of haemostatic platelet aggregation associated with inhibition of the release reaction by ASA (18). That the loss of blood was already greater in the first two minutes suggests that the drug does not affect reversal of vasoconstriction, but that its inhibitory effect on platelet aggregation becomes effective within that time. The conclusion is compatible with observations (19) indicating that the release reaction begins to manifest itself significantly later than about 15 but certainly within the first minute after injury.

What our observations suggest is that primary haemostasis depends on a sequence of processes highly integrated with respect to time.

The results reported here together with earlier ones (7), support the conclusion already expressed that the delay in primary haemostasis produced by a diet of fatty fish is due to a mechanism different from that produced by ASA, notwithstanding apparent similarities in their effects on platelet aggregation in vitro (data will be published) and on the bleeding time.

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We wish to thank Miss Elisabeth Nilsson, Mrs. Ingrid Svenstam, Mrs. Gertrud Wohlfart and Mrs. Sylvie Persson for technical assistance; and the staff of the hospital canteen for preparing the food; Faculty of Medicine, University of Lund; and the Swedish Medical Research Council (Project B82-19X-05701-03); the British Heart Foundation, and the Nutritional Foundation of the Olemargarine Industry in Sweden for generous financial support.

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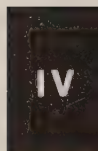
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Paper IV



## THROMBOXANE A<sub>2</sub> IN SKIN-BLEEDING-TIME BLOOD AND IN CLOTTED VENOUS BLOOD BEFORE AND AFTER ADMINISTRATION OF ACETYLSALICYLIC ACID

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**Summary**      The 'Simplate' technique for measuring skin bleeding time was adapted to quantify thromboxane A<sub>2</sub> in the emerging blood as the stable degradation product thromboxane B<sub>2</sub> in twelve Swedish and ten English volunteers. During the bleeding time thromboxane B<sub>2</sub> concentrations increased, but as the rate of blood loss fell the rate of production of thromboxane A<sub>2</sub> was constant. The English subjects had shorter bleeding times and produced more thromboxane A<sub>2</sub> than the Swedish subjects. When the Swedish subjects were grouped according to bleeding times those with the shortest had more thromboxane A<sub>2</sub> than those with longer bleeding times. Clotting venous blood in vitro produced much more thromboxane A<sub>2</sub> than bleeding-time blood and there was no correlation with bleeding time. Determination of the capacity of clotting blood to form thromboxane A<sub>2</sub> is therefore irrelevant to in-vivo haemostasis. Acetylsalicylic acid greatly diminished the appearance of thromboxane A<sub>2</sub> in the bleeding time and prevented the increase of thromboxane A<sub>2</sub> concentration with time.

### Introduction

THE extent to which physiological haemostasis depends on prostaglandins and related endogenous agents is still uncertain. Inhibition of prostaglandin biosynthesis by acetylsalicylic acid is associated with increases in bleeding time.<sup>1-3</sup> Some familial bleeding tendencies are associated with deficiencies in the synthesis of thromboxane A<sub>2</sub> by platelets.<sup>4</sup> Neither of these raise bleeding time by more than about twice. Studies in human beings given thromboxane-A<sub>2</sub>-synthetase inhibitors indicate that haemostasis depends only to a minor extent on thromboxane A<sub>2</sub>.<sup>5</sup>

We have already shown that the impairment of human haemostasis by acetylsalicylic acid or by a diet enriched with fish, both of which diminish prostaglandin formation, occurs by different mechanisms.<sup>6,7</sup> To determine the contribution of thromboxane A<sub>2</sub> to primary haemostasis of healthy human beings in vivo, we measured thromboxane A<sub>2</sub> (as its stable metabolite thromboxane B<sub>2</sub>) in blood coming from standard incisions made for determining skin bleeding time and the thromboxane A<sub>2</sub> produced during in-vitro clotting of venous blood from the same individuals, determined before and after they ingested one dose of acetylsalicylic acid.

## Subjects and Methods

The subjects were twelve apparently healthy Swedish male volunteers (mean age  $29 \pm 1$  SEM years) who had taken no drugs for at least 2 weeks before these investigations. After an overnight fast for 12 h the bleeding time was determined on one arm. From a similar incision on the other arm blood was collected for thromboxane  $B_2$  determinations. Thromboxane  $B_2$  was also determined in a blood sample from the antecubital vein of that arm. A fortnight later the determinations were repeated before and 2 h after oral administration of a single dose of acetylsalicylic acid (5 mg/kg body-weight). In ten similar English subjects, bleeding times and thromboxane  $B_2$  levels in bleeding-time blood were determined once in the same way. Bleeding times were determined by the same observer in all the subjects.

Bleeding times were determined with the standard 'Simplate I' device (General Diagnostics Inc).<sup>8,9</sup> The blood emerging from the skin incision was removed quantitatively every 30 s with a disc of filter paper (Whatman no 1) until bleeding stopped. The bleeding-time-blood volumes were measured by eluting the spots of blood from the filter paper into Drabkin's solution as previously described.<sup>7</sup> In each eluate the haemoglobin concentration was determined spectrophotometrically and the corresponding blood volume obtained from standard curves. The results were expressed as  $\mu$ l of blood emerging in each 30 s period.

Thromboxane  $A_2$  concentrations in the bleeding-time blood were determined by radioimmunoassay of the stable degradation product thromboxane  $B_2$ . The blood emerging from skin incisions made by the simplate I device was collected in 1 min periods into capillary tubes containing known volumes of a mixture of 100 mmol/l ethylenediamine tetra-acetic acid and 5.59 mmol/l indomethacin to inhibit in-vitro formation of thromboxane  $B_2$ . After mixing, the tubes were centrifuged at 15 000 *g* for 2 min. The clear supernatants were removed, frozen, stored at  $-20^\circ\text{C}$ , and assayed later for thromboxane  $B_2$ . The thromboxane  $B_2$  standard (U51541 lot 0071-CLM-059C) and the antibodies against thromboxane  $B_2$  (21) were kindly supplied by Dr John Pike of the Upjohn Company, Kalamazoo, USA, and Dr John Vane of the Wellcome Foundation Ltd, Beckenham, UK, respectively. The total amounts of thromboxane  $B_2$  formed in the bleeding-time blood were calculated for each individual by summing the products of the concentrations and the volumes of successive blood samples. Thromboxane  $B_2$  formation was determined also in the serum from blood collected by venepuncture into glass tubes in which it was made to clot by incubation at  $37^\circ\text{C}$  for 1 h. The tubes were centrifuged at 2000 *g* for 10 min, the supernatant sera separated, frozen, stored at  $-20^\circ\text{C}$ , and assayed for thromboxane  $B_2$  by the same procedure.

The results were evaluated statistically by Student's *t* test.

## Results

### *Bleeding Times*

In the twelve Swedish subjects the bleeding time was significantly longer than that in the ten English subjects ( $8.1 \pm 0.5$  min *vs*  $5.6 \pm 0.4$  min;  $p < 0.001$ ).

### *Thromboxane $B_2$ Formation in Bleeding-time Blood*

The variability in the rate of appearance of thromboxane  $B_2$  in the bleeding-time blood of one subject was determined.

The mean standard error of six determinations was 0.1 ng/ml, which establishes the necessary reproducibility.

In one of the Swedish subjects the bleeding-time blood contained almost no measurable thromboxane  $B_2$  at any time. There was no reason to suppose that this was caused by unreported medication, but results for this subject were omitted from the analyses.

In the other eleven Swedish subjects the bleeding-time blood contained thromboxane  $B_2$  in concentrations which increased almost linearly ( $r=0.69$ ;  $p<0.001$ ) from the 1st to the 5th minute, both in the group as a whole (fig 1) and in most individuals. There were considerable differences in the thromboxane  $B_2$  concentration between individuals (see table). After 5 min too little blood was coming from the cuts for accurate measurement and thromboxane  $B_2$  assay.

We compared thromboxane  $B_2$  concentrations in the bleeding-time blood of the six subjects with the longest bleeding times with that of the five with the shorter bleeding times. These five individuals (bleeding times less than 7.5 min) had significantly ( $p<0.05$ ) higher concentrations of thromboxane  $B_2$  in the bleeding-time blood at most time points than those with longer bleeding times.

In the English subjects the increase in thromboxane  $B_2$  concentration in the bleeding-time blood with time was

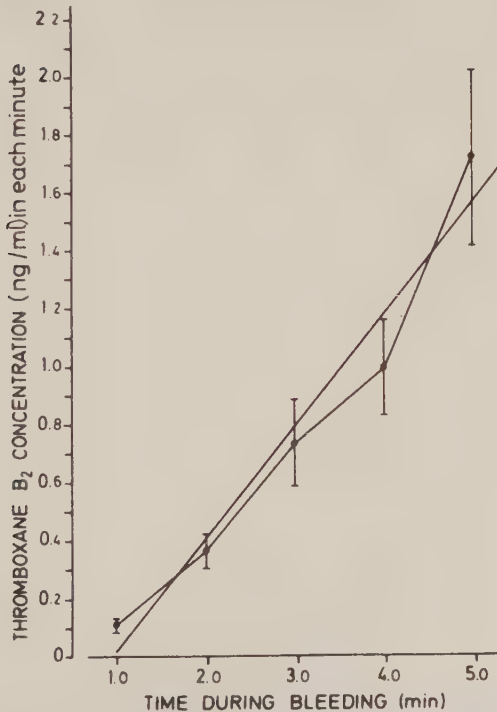


Fig 1—Increase in mean  $\pm$  SEM thromboxane  $B_2$  concentration in bleeding-time blood with time in eleven Swedish subjects and its regression line ( $r=0.69$ ;  $p<0.001$ ).



RANGE OF THROMBOXANE B<sub>2</sub> CONCENTRATIONS (TXB<sub>2</sub>) IN  
BLEEDING-TIME BLOOD OF SWEDISH SUBJECTS WITH  
INCREASING TIME

| Duration<br>of bleeding<br>(min) | TXB <sub>2</sub> range (ng/ml) |
|----------------------------------|--------------------------------|
| 1.0                              | 0.02-0.24                      |
| 2.0                              | 0.08-0.70                      |
| 3.0                              | 0.13-1.64                      |
| 4.0                              | 0.30-1.71                      |
| 5.0                              | 0.55-3.78                      |

similar to that of the Swedish subjects, but the concentrations were significantly greater ( $p < 0.05$ ) at all times (fig 2).

The total amount of thromboxane B<sub>2</sub> produced per minute in the bleeding-time blood of the Swedish subjects rose progressively during the first 3 min and was thereafter constant for as long as assays were possible (fig 3). There was no correlation between the rate of thromboxane B<sub>2</sub> production and the bleeding time ( $r = 0.02$ ).

*Thromboxane B<sub>2</sub> Formation in Clotting Venous Blood*

The Swedish subjects' clotted venous blood contained  $175 \pm 25$  ng thromboxane B<sub>2</sub>/ml. There was no relation between the bleeding time and the concentration of thromboxane B<sub>2</sub> in clotting venous blood. To evaluate the

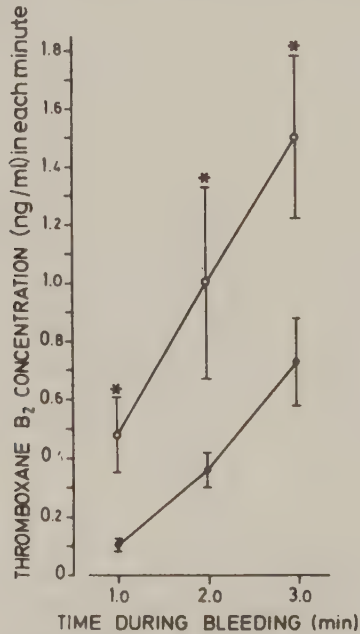


Fig 2—Increase in mean  $\pm$  SEM thromboxane B<sub>2</sub> concentration in bleeding-time blood with time in eleven Swedish (●—●) and ten English subjects (○---○).

\* $p < 0.05$ .

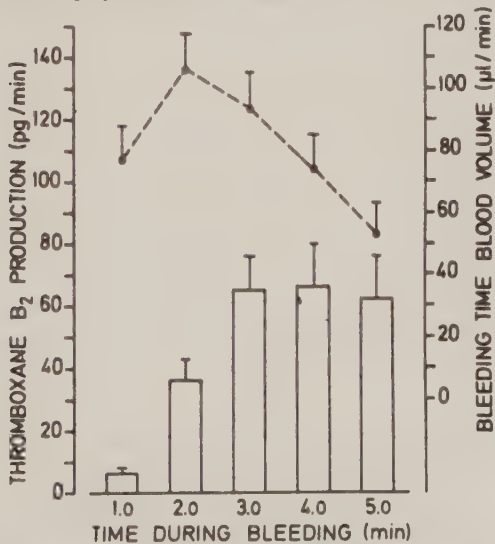
relevance, if any, of thromboxane  $A_2$  concentrations in clotted venous blood to the appearance of thromboxane  $A_2$  in blood from a haemostatic site, the total amounts of thromboxane  $B_2$  formed in bleeding-time blood during the first 5 min were calculated for each individual. The total amounts of thromboxane  $B_2$  were also calculated for the same volumes of blood clotting in vitro. The amount of thromboxane  $A_2$  formed by blood clotting in vitro exceeded by two orders of magnitude that produced in blood during haemostasis in skin incisions ( $74.8 \pm 12.3$  ng vs  $0.23 \pm 0.03$  ng).

#### *Effect of Acetylsalicylic Acid*

When the Swedish subjects took a single dose of acetylsalicylic acid (5 mg/kg body-weight) their bleeding time increased by 124% to  $18.9 \pm 2.6$  min. Thromboxane  $B_2$  levels in the bleeding-time blood were greatly diminished, but small, measurable concentrations persisted and did not rise with time (fig 4). After acetylsalicylic acid the concentration of thromboxane  $B_2$  in clotted venous blood was reduced to  $0.59 \pm 0.03$  ng/ml.

#### **Discussion**

Our aim was to find out the extent to which primary haemostasis in man depends on thromboxane  $A_2$ . There are many reports about the presence of thromboxane  $A_2$  in circulating blood and about its formation when blood clots in vitro. However, we believe that these reports are irrelevant in evaluating the contribution of thromboxane  $A_2$  to haemostasis at injury sites—ie, to the process in which it is claimed to play a dominant role.<sup>10,11</sup>



**Fig 3**—Mean $\pm$ SEM rate of thromboxane  $B_2$  production in blood coming from bleeding-time skin incisions of eleven Swedish volunteers in successive 1 min periods.

Our results show that in blood coming from skin incisions standardised for bleeding-time determinations thromboxane  $A_2$  concentrations increased linearly for as long as enough blood emerged for the determinations, but that the amounts of thromboxane  $A_2$  produced were far smaller than those produced by the same volume of venous blood clotting *in vitro*. If the haemostatic process in the skin incisions depended upon thromboxane  $A_2$ , one might therefore expect that small variations in the amounts formed would be accompanied by corresponding variations in the bleeding time. This expectation was borne out by two of our observations: the English subjects had shorter bleeding times and more thromboxane  $A_2$  in the bleeding-time blood than the Swedish subjects (fig 2) and the Swedish subjects with the shorter bleeding times had higher thromboxane  $A_2$  concentrations than those with the longer bleeding times. Both findings indicate a connection between the duration of bleeding and the capacity of the local haemostatic mechanism, presumably the plug of platelets, to produce thromboxane  $A_2$ . Nevertheless, they are also compatible with the possibility that thromboxane  $A_2$  is a measure of the amount of platelet aggregation rather than its cause.

The rate of blood loss from standard skin incisions rose for about 2 min, after which it fell continuously until bleeding stopped. We have shown that this pattern occurs regardless of

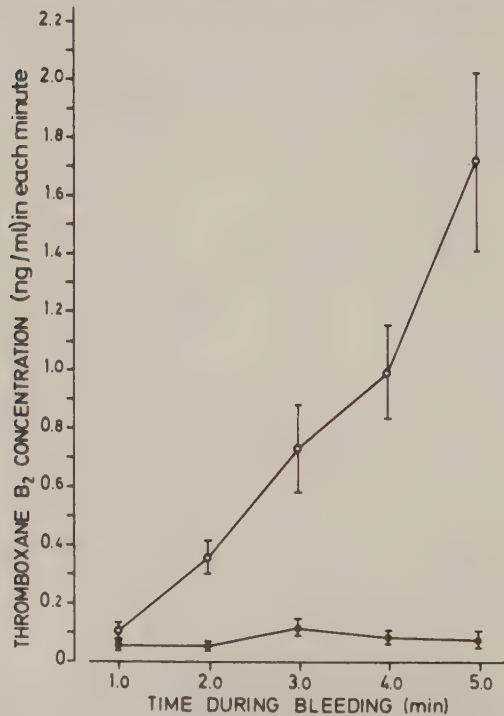


Fig 4—Mean  $\pm$  SEM thromboxane  $B_2$  concentration in bleeding-time blood before (○---○) and after (●---●) acetylsalicylic acid.

the final bleeding time, but that the quantity of blood emerging from the time of incision up to the maximum is correlated significantly with the final bleeding time.<sup>7</sup>

It is apparent that thromboxane  $B_2$  concentrations can rise as bleeding proceeds when the amounts of blood from the incisions fall. Indeed, thromboxane  $B_2$  concentrations increased progressively, but the rate of production rose only during the first 3 min and thereafter remained constant, at about 60 pg per min (fig 3). This finding supports our previous conclusion that the growing plug of platelets produces increasing amounts of thromboxane  $A_2$  only during an earlier stage of the haemostatic process. Since only a very small fraction of the total thromboxane-producing capacity of blood is utilised at sites of injury, we can conclude that only a small proportion of platelets at risk become involved, at least as far as this component of haemostasis is concerned.

Clotting venous blood produced two orders of magnitude more thromboxane  $A_2$  than the same volume of bleeding-time blood; it is not surprising, therefore, that there was no relation between the bleeding time and the formation of thromboxane  $A_2$  in blood clotting *in vitro*. Thus, determinations of the capacity of clotting blood to form thromboxane  $A_2$  are not relevant to *in-vivo* haemostasis. Despite the correlation in appropriate groups of subjects between bleeding time and thromboxane  $B_2$  concentration in bleeding-time blood, in individuals the bleeding time was not correlated significantly with the production rate or the total production of thromboxane  $B_2$ . There was also no correlation between bleeding time and thromboxane  $B_2$  in the rising phase of bleeding, in which the total amount of blood loss is related to the bleeding time. Therefore, the local formation of thromboxane  $A_2$  is not the only, or indeed the major, determinant of skin bleeding time, which is the most reproducible parameter of haemostatic effectiveness in general use.

After the subjects had taken a single dose of acetylsalicylic acid the appearance of thromboxane  $A_2$  in bleeding-time blood was greatly diminished but not abolished (fig 4). Although thromboxane  $A_2$  was still produced, there were no increases in concentration and total production of thromboxane  $A_2$  with time. Whether the small persistent production could have been eliminated by larger doses of acetylsalicylic acid is uncertain; the administered dose was that which in previous experiments<sup>12</sup> had produced the greatest increase in bleeding time.

It is instructive to consider results for some of the individuals. One subject produced little thromboxane  $A_2$  in the first 3 min; acetylsalicylic acid caused no decrease in his thromboxane  $A_2$  concentration but raised the bleeding time to over 40 min (ie, by 281%). Another subject produced much more thromboxane  $A_2$ ; acetylsalicylic acid decreased its production almost to zero and raised the bleeding time to 10.5 min (ie, by only 61%). These, and our other results,

confirm that primary haemostasis does not depend only or even mainly on local formation of thromboxane  $A_2$ .

There is strong evidence from animals and healthy human volunteers that the agent mainly responsible for primary haemostasis *in vivo* is ADP.<sup>13</sup> Our new findings, together with that evidence, suggest that the haemostatic process measured by skin bleeding time involves locally formed thromboxane  $A_2$  but is not dependent on it. The probable sequence of events is local release of ADP from damaged cells (unpublished) causing activation, adhesion, and aggregation of a minor proportion of arriving platelets; formation and release of thromboxane  $A_2$  by these platelets; and continuing activation by both agents of a minor proportion of platelets at risk in the bleeding-time blood until their aggregates suffice to occlude the opened vessels.

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Paper V





DELAY IN PRIMARY HAEMOSTASIS PRODUCED BY A FISH DIET  
WITHOUT CHANGE IN LOCAL THROMBOXANE A<sub>2</sub>

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## ABSTRACT

Twelve apparently healthy male volunteers were maintained for six weeks on a fish diet, the haemostatic effects of which were determined as standardised skin bleeding times; thromboxane A<sub>2</sub> formation in vivo in the bleeding time blood and in vitro in clotting venous blood; platelet aggregability; and phospholipids.

The fish diet within one week changed the fatty acid composition of platelet membrane phospholipids, with increases in  $\omega$ -3 and decreases in  $\omega$ -6 fatty acids; decreased platelet aggregability; but left bleeding time unchanged. Not until six weeks on the diet did bleeding time increase (by 37%;  $p < 0.01$ ) and it was still increased three weeks after the volunteers had resumed a normal diet and the platelet fatty acid composition was normal again. Therefore, the effects of the diet on in vitro aggregation or on the fatty acid composition of platelets did not accompany its effect on the bleeding time. The diet caused no changes in the in vivo appearance of thromboxane A<sub>2</sub> in the bleeding time blood, contrasting with its effect in decreasing the in vitro formation of thromboxane A<sub>2</sub> in clotting venous blood.

These observations suggest that such fish diets do not delay haemostasis by diminishing the formation of thromboxane A<sub>2</sub> locally nor directly by the increase of dietary  $\omega$ -3 fatty acids in the platelets.

## INTRODUCTION

Atherosclerotic vascular disease and its complications are the predominant cause of morbidity and mortality in the populations of most developed countries. Great interest has therefore been aroused by the claim that the clinical manifestations of this disease are rare in Greenland Eskimos as well as in attempts to establish possible reasons for this<sup>1</sup>. A major hypothesis proposes that Eskimos benefit from eating more fish and marine mammals, and specifically from having thereby an extraordinarily high intake of marine fatty acids of the  $\omega$ -3 family, mainly eicosapentaenoic acid (EPA), in their diet<sup>2, 3</sup>. The proposition is that atherogenesis depends on interactions between arterial walls and circulating platelets; and that EPA reduces this by replacing arachidonic acid in platelets whereby their aggregability is diminished and bleeding time is increased<sup>3, 4</sup>.

Indeed, when normal European diets are supplemented with fish<sup>5, 6</sup> or fish oil<sup>7, 8</sup> EPA substitutes for arachidonic acid in the phospholipids of platelet membranes. We recently reported that the associated decrease in platelet aggregability and increase in bleeding time are similar to those caused by acetylsalicylic acid (ASA)<sup>5, 9</sup>. In our current attempt to find out the mechanism(s) responsible for these fish diet effects we have already provided evidence that ASA and diets rich in  $\omega$ -3 polyunsaturated fatty acids delay primary haemostasis by different mechanisms<sup>5, 9, 10</sup>.

We now report further analyses of the effects of fish-supplemented diets on haemostasis, in particular on the local formation of thromboxane  $A_2$  in blood collected from standardised skin incisions made for bleeding time determinations. The result provide more evidence for doubting that fish diets delay haemostasis by diminishing

thromboxane  $A_2$ , as currently commonly claimed; and that the delay depends directly on the abundance of  $\omega$ -3 fatty acids in the diet.

## METHODS

### Subjects

The subjects were 12 apparently healthy Swedish male volunteers aged  $26 \pm 1$  years (mean  $\pm$  s.e.m.). All were non-smokers who took no drugs for one month before and none during the investigation. For the experimental period of six weeks the volunteers continued on their normal Swedish diet with the only change being a supplement of approximately 150 g of fatty fish, mainly herring, salmon and mackerel, per day. This diet, which was well tolerated, increased the intake of EPA (C20:5,  $\omega$ -3) by 2-3 g per day.

Duration of bleeding from standardised skin incisions, i.e. bleeding times; bleeding time blood volumes; thromboxane  $B_2$  concentrations in bleeding time blood and in clotted venous blood; in vitro platelet aggregation and platelet and plasma lipid analyses were determined at the following times: before the diet on two occasions two weeks apart (the tables and figures show means of these two determinations); during the diet after one, three and twice after six weeks (shown as means of these two determinations); and three and eight weeks after the end of the diet. Bleeding times and platelet aggregations were determined again 14 weeks after the end of the diet. Values shown for one week on the diet and eight and 16 weeks after the end of the diet are means of determinations from 11, 10 and 10 volunteers respectively.

The volunteers abstained from alcohol for three days before blood sampling which was in the morning after fasting for 12 hours overnight.

## Lipid analysis

Platelets and plasma were prepared for lipid analysis according to a method already described with minor modifications<sup>11</sup>. Extraction was with chloroform-methanol 2:1 or 1:1 for platelets and plasma respectively in the presence of butylated hydroxytoluene. Platelet phospholipids were separated by thin layer chromatography on 0.5 mm Silica gel with chloroform-methanol-acetic acid-water (65:25:4:4 v/v). The separated fraction containing phosphatidyl choline (PC) and including phosphatidyl inositol and phosphatidyl serine was methylated and analysed for fatty acids by gas-liquid chromatography on a Varian 3700 equipped with a flame-ionisation detector on a 200 cm glass column (2 mm internal diameter) packed with 15% diethylene/glycol/succinate (DEGS) coated on chromosorb W, 80-100 mesh. The column was operated at 190<sup>o</sup> with nitrogen as carrier gas. Individual esters were identified by comparison with standards and of phospholipid fatty acid methylester from bull testis. Peaks were quantified and the fatty acid distributions estimated by a computer CDS 111 (with 17:0 heptadecanoic acid as internal standard).

## Quantification of haemostasis

Bleeding times<sup>12</sup> were determined with a standard Simplate II device from General Diagnostics<sup>13</sup>. The skin incision was blotted every 30 sec with a disk of filter paper (Whatman No. 1). To quantify bleeding time blood volumes, the blood spots were eluted in Drabkin solution as already described<sup>10</sup>. In each eluate the haemoglobin concentration was estimated spectrophotometrically and converted to the corresponding blood volume from standard curves. Results were expressed as microlitre blood emerging in each 0.5 min period.

Platelets were counted in ultra flow 100 (Clay Adams).



Thromboxane A<sub>2</sub> concentrations in the bleeding time blood were determined by radioimmunoassay of the stable degradation product thromboxane B<sub>2</sub>. The blood emerging from skin incisions made by the Simplate I device was collected in one-minute periods into capillary tubes containing known volumes of a mixture of 100 mM EDTA and indomethacin at 5.6 mM to inhibit formation of thromboxane B<sub>2</sub> in vitro. After mixing the tubes were centrifuged at 15,000 g for 20 min. The clear supernatants were removed, frozen and stored at -20°C until assayed for thromboxane B<sub>2</sub>. The thromboxane B<sub>2</sub> standard (U51541 lot 0071-CLM-059C) and the antibodies against thromboxane B<sub>2</sub> were kindly supplied by Dr. John Pike of the Upjohn Company in Kalamazoo, U.S.A. and Dr. John Vane of the Wellcome Foundation Ltd., Beckenham, U.K., respectively. The total amounts of thromboxane B<sub>2</sub> formed in the bleeding time blood were calculated for each individual by summing the products of the concentrations in successive blood samples and their volumes. Thromboxane B<sub>2</sub> formation was determined also in the serum from blood collected by venepuncture into glass tubes in which it was made to clot by incubation at 37°C for 1 h. The tubes were centrifuged at 2,000 g for 10 min, the supernatant sera separated, frozen at -20°C and assayed for thromboxane B<sub>2</sub> by the same procedure.

#### Platelet aggregation

Venous blood from fasting subjects was centrifuged at 275 g at room temperature for 10 min to produce platelet-rich plasma. The red cell fraction was re-centrifuged at 2,600 g for 20 min to produce platelet-poor plasma. Platelet aggregation was measured photometrically<sup>14</sup> in a Payton aggregometer. The aggregating agents were collagen (Hormon-Chemie, Munich) at 1.0, 2.0, 4.0 and 10.0 µg/l; adenosine diphosphate (Sigma, St. Louis, U.S.A.) at 0.5, 1.0, 2.0, 3.0, 4.0, 10.0 and 30.0 mmol/l.

The indices of aggregation were: (1) maximum decrease in optical density expressed as a percentage of the difference in optical density between platelet-rich and platelet-poor plasma; (2) maximum velocity arbitrarily expressed in mm/min; and (3) the lowest concentration of ADP which induced secondary aggregation, i.e. the release reaction. This concentration is referred to as the threshold value of ADP.

Statistical evaluation of lipid analysis, and of thromboxane  $B_2$  concentrations in bleeding time blood and in clotted venous blood was by Student's t-test and of bleeding times and platelet aggregation by Wilcoxon's test.

## RESULTS

### Platelet and plasma lipids

The fish diet rich in  $\omega$ -3 fatty acids produced changes in the relative fatty acid composition of the phosphatidylcholine, i.e., lecithin, of plasma and platelets (Table 1). Already after one week the proportion of  $\omega$ -3 polyunsaturated fatty acids in both platelet and plasma phosphatidylcholine increased ( $p < 0.001$ ), due to an increase of both eicosapentaenoic acid (C20:5) docosahexaenoic acid (C22:6); these changes persisted throughout the diet. Three weeks after the end of the diet these acids were still a little raised and after eight weeks the fatty acid composition was essentially as before the diet. The proportion of  $\omega$ -6 fatty acids fell after one week ( $p < 0.001$ ): in the platelets mainly due to a decrease in arachidonic acid (C20:4) but also in linoleic acid (C18:2); and in the plasma because of a decrease in linoleic acid but after 3 weeks also in arachidonic acid. These changes resulted in a three-fold increase in the ratio of C20:5/C20:4 in the platelets. The effects of the diet on the changes in the proportions of the polyunsaturated fatty acids were similar in plasma and platelets (Table 1). Three and 8 weeks after the end of the diet the fatty acid composition of both plasma and platelet lecithin had reverted almost entirely to the pre-dietary pattern.

Table 1 Fatty acid composition (per cent) in platelet phosphatidyl choline (PC) before, during and after the fish diet.

|                                                                      | 16:0           | 18:0             | 18:1            | 18:2            | 20:3            | 20:4             | 20:5            | 22:5            | 22:6            | Total           | Total            |
|----------------------------------------------------------------------|----------------|------------------|-----------------|-----------------|-----------------|------------------|-----------------|-----------------|-----------------|-----------------|------------------|
|                                                                      |                |                  |                 | ω-6             | ω-6             | ω-6              | ω-3             | ω-3             | ω-3             | ω-3             | ω-6              |
| <i>Baseline</i>                                                      |                |                  |                 |                 |                 |                  |                 |                 |                 |                 |                  |
| 0 weeks                                                              | 19.9<br>(0.6)  | 22.7<br>(0.2)    | 22.6<br>(0.2)   | 7.0<br>(0.2)    | 1.6<br>(0.1)    | 18.3<br>(0.4)    | 0.6<br>(0.1)    | 0.9<br>(0.1)    | 1.5<br>(0.1)    | 4.3<br>(0.2)    | 28.9<br>(0.5)    |
| <i>Dietary period</i>                                                |                |                  |                 |                 |                 |                  |                 |                 |                 |                 |                  |
| 1 weeks                                                              | 20.4<br>(0.3)  | 22.2**<br>(0.2)  | 22.5<br>(0.2)   | 6.1***<br>(0.2) | 1.4*<br>(0.1)   | 17.0**<br>(0.3)  | 1.7***<br>(0.2) | 1.0***<br>(0.1) | 2.1***<br>(0.2) | 6.4***<br>(0.4) | 26.2***<br>(0.5) |
| 3 weeks                                                              | 20.8<br>(0.3)  | 21.9***<br>(0.2) | 22.9<br>(0.2)   | 6.5***<br>(0.2) | 1.3***<br>(0.1) | 16.3***<br>(0.4) | 1.7***<br>(0.1) | 1.1***<br>(0.1) | 2.3***<br>(0.2) | 6.5***<br>(0.3) | 25.8***<br>(0.4) |
| 6 weeks                                                              | 20.9*<br>(0.2) | 21.7***<br>(0.3) | 23.4**<br>(0.2) | 6.2***<br>(0.2) | 1.3***<br>(0.1) | 15.6***<br>(0.4) | 2.0***<br>(0.2) | 1.1***<br>(0.1) | 2.5***<br>(0.2) | 7.2***<br>(0.3) | 24.6***<br>(0.5) |
| <i>Post dietary period</i>                                           |                |                  |                 |                 |                 |                  |                 |                 |                 |                 |                  |
| 3 weeks                                                              | 20.0<br>(0.3)  | 22.4**<br>(0.3)  | 22.9<br>(0.2)   | 7.1<br>(0.2)    | 1.6<br>(0.1)    | 17.2*<br>(0.4)   | 0.9***<br>(0.1) | 1.0**<br>(0.1)  | 1.9***<br>(0.1) | 5.1***<br>(0.2) | 27.8*<br>(0.4)   |
| 8 weeks                                                              | 19.9<br>(0.3)  | 22.7<br>(0.3)    | 22.8<br>(0.2)   | 6.7<br>(0.3)    | 1.6<br>(0.1)    | 18.0<br>(0.4)    | 0.9**<br>(0.1)  | 0.9<br>(0.1)    | 1.8**<br>(0.2)  | 4.8*<br>(0.3)   | 28.1<br>(0.5)    |
| Correlation co-efficient with corresponding values for plasma PC are | 0.22*          | 0.40***          | 0.04            | 0.71***         | 0.44***         | 0.57***          | 0.92***         | 0.33**          | 0.65***         | 0.84***         | 0.83***          |

Upper values are means, lower values s.e.m. Significance of differences

\* p < 0.05 \*\* p < 0.01 \*\*\* p < 0.001

Bleeding times

Before dieting began the mean bleeding time for both determinations in all the volunteers was  $8.1 \pm 0.5$  min. This was not increased significantly after one and three weeks on the fish diet, although two volunteers after dieting for one week bled for more than 20 minutes; their values were omitted. After six weeks on the diet the bleeding time increased in all volunteers to  $10.9 \pm 0.6$  min, i.e. by 37%. Although there were considerable differences between individuals (range 2-93%), the mean increase was highly significant ( $p < 0.01$ ) (Fig. 1). Three weeks after the end of the diet the bleeding time was still significantly increased, but no longer after 8 and 16 weeks on the normal diet (Fig. 1). There were no significant changes in the concentration of platelets in the peripheral blood during the diet compared with before and after.

Platelet lipid composition

|                     |      |      |      |      |      |      |
|---------------------|------|------|------|------|------|------|
| C 20 : 5            | 0.6  | 1.7  | 1.7  | 2.0  | 0.9  | 0.9  |
| C 20 : 4            | 18.3 | 17.0 | 16.3 | 15.6 | 17.2 | 18.0 |
| Ratio $\times 10^3$ | 33   | 100  | 104  | 128  | 52   | 50   |

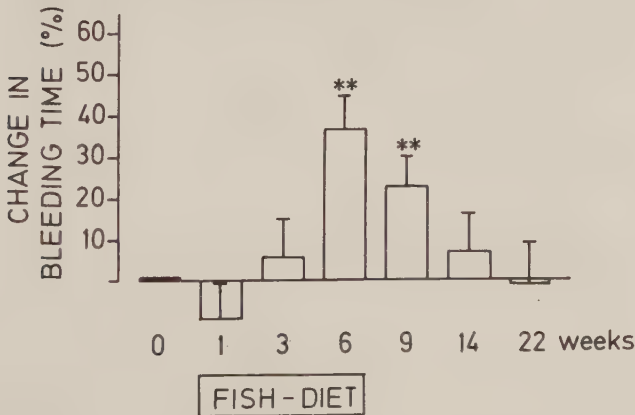


Fig. 1. Effect of the fish-diet on in-vivo bleeding time and platelet lipid composition.

### Thromboxane A<sub>2</sub> in the bleeding time blood

In one of the volunteers the bleeding time blood contained almost no measurable thromboxane B<sub>2</sub> throughout without any reason for supposing that this was due to unreported medication; this volunteer was omitted from the group. In the other eleven volunteers the day-to-day variation of the mean thromboxane B<sub>2</sub> concentration varied little within the group. (Fig. 2). In the predietary controls thromboxane B<sub>2</sub> concentrations increased almost linearly from the first to the fifth minutes (Fig. 2). After five minutes; the amount of blood coming from the cuts were too small for accurate quantification.

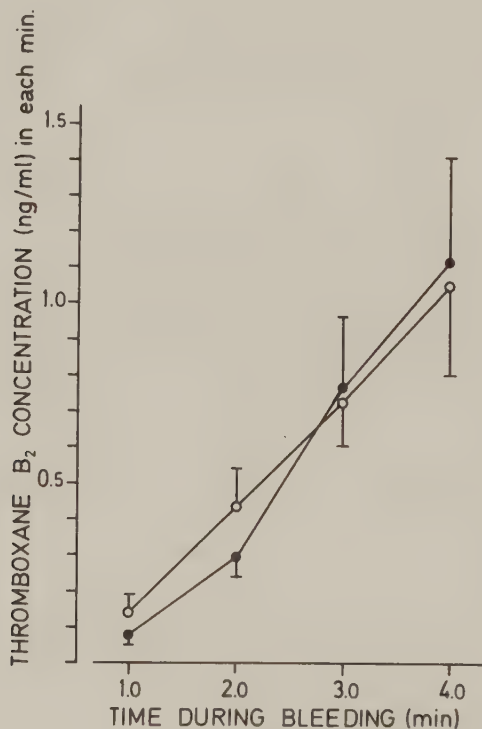


Fig. 2. Day-to-day variability (n=2) of the appearance of thromboxane B<sub>2</sub> in the bleeding time blood in the 11 Swedish volunteers (mean  $\pm$  s.e.m.)

After one and three weeks on the fish diet; when the bleeding time was not yet prolonged, there were no significant changes in the thromboxane  $B_2$  concentrations in the bleeding time blood (Fig. 3). After six weeks when the bleeding time was prolonged there were still no changes in the thromboxane  $B_2$  nor again 3 and 8 weeks after the end of the diet.

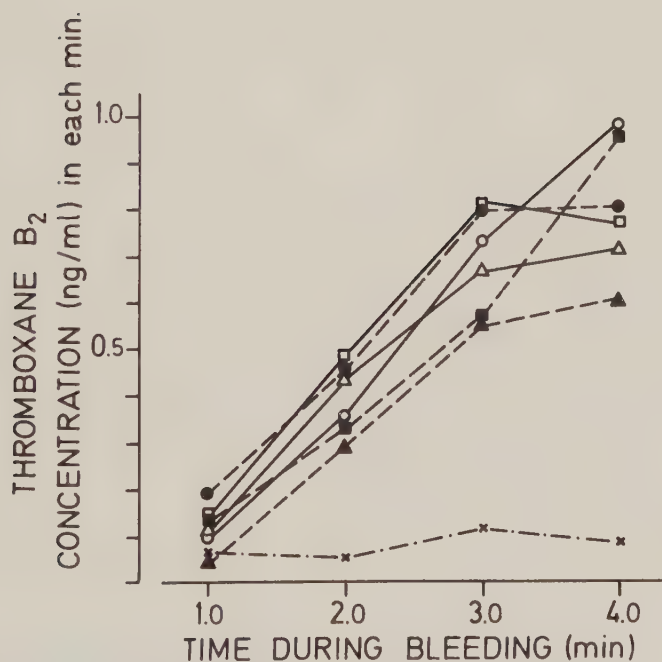


Fig. 3. Thromboxane  $B_2$  concentrations (mean  $\pm$  s.e.m) in the bleeding time blood before (0—0) and after (x---x) one dose of 5 mg/kg b.w. ASA (from Thorngren et al 1983) and after 1 (▲---▲), 3 (■---■) and 6 weeks (●---●) of the fish-diet and 3 (△---△) and 8 weeks (□---□) after the end of the diet.



The total amount of thromboxane B<sub>2</sub> produced per minute in the bleeding time blood increased progressively during the first three minutes and was thereafter constant for as long as the determination could be made. The rate of production was unchanged during the diet (Fig. 4). Even in the six individuals whose bleeding times were increased most by the diet, the rate of thromboxane production was not significantly changed.

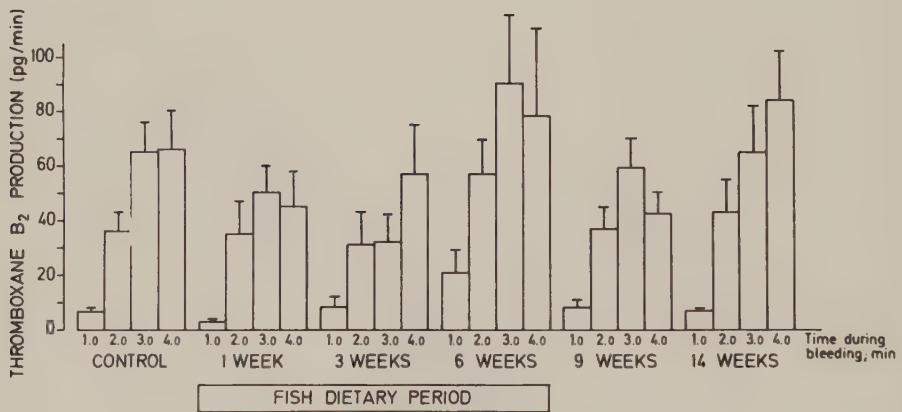


Fig. 4. Rate of thromboxane B<sub>2</sub> production (mean + s.e.m.) in blood coming from bleeding time skin incisions (Simplate II) before and during the fish diet.

### Thromboxane A<sub>2</sub> Formation in Clotted Venous Blood

In blood taken before the diet, thromboxane B<sub>2</sub> formation was  $168 \pm 23$  ng/ml. After one week of the diet, this was already significantly decreased and remained decreased during and for at least eight weeks after the diet (Table 2).

TABLE 2

Total amount of thromboxane B<sub>2</sub> released, from platelets adjusted to a platelet concentration of  $2 \times 10^8$ /ml in serum from clotted venous blood, before, during and after the fish diet.

|                                      | THROMBOXANE B <sub>2</sub><br>produced (ng/ml) |                     |                    |                                         |
|--------------------------------------|------------------------------------------------|---------------------|--------------------|-----------------------------------------|
| Control before<br>the diet<br>0 week | During the diet                                |                     |                    | Control after<br>the diet               |
|                                      | 1 week                                         | 3 weeks             | 6 weeks            | 3 weeks 8 weeks                         |
| 168 <sub>±</sub> 23                  | 121 <sub>±</sub> 23                            | 112 <sub>±</sub> 21 | 97 <sub>±</sub> 22 | 117 <sub>±</sub> 17 128 <sub>±</sub> 26 |
| Difference                           | *                                              | *                   | **                 | * **                                    |

Values given are mean + s.e.m. significance of difference from control values \*  $\bar{p} < 0.05$  \*\*  $p < 0.01$

### Platelet aggregation

Platelet aggregation induced by collagen measured as maximum velocity was not changed by the diet but; when measured as maximum change in optical density, aggregation was significantly decreased after one week on the diet ( $p < 0.01$ ) but not subsequently except for the highest concentrations (10  $\mu$ l/ml) of collagen for which it remained decreased until the end of the diet.

Aggregation by ADP measured as maximum velocity decreased significantly after three weeks on the diet and remained decreased not only during the diet but for at least 16 weeks after its end ( $p < 0.02$ ) (Fig. 5). Platelet aggregability measured as maximum optical density change decreased similarly.

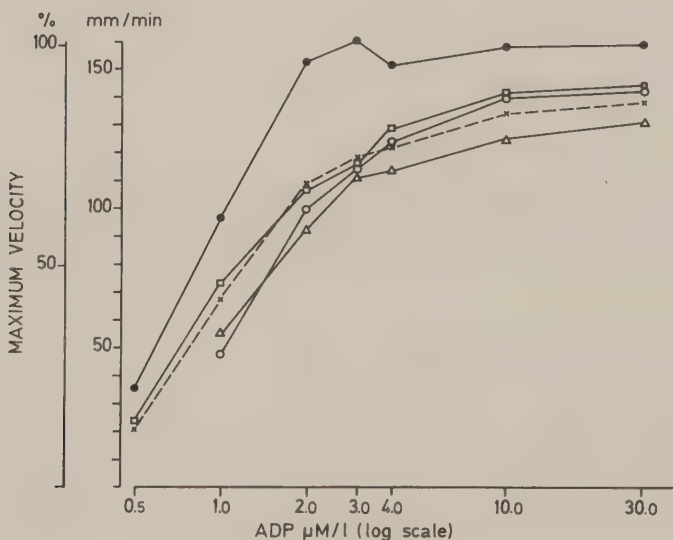


Fig.5. Platelet aggregation (mean maximum velocity) by ADP before (●—●), after 1 (○—○), 3 ( $\Delta$ — $\Delta$ ) and 6 weeks ( $\square$ — $\square$ ) of the fish-diet and 16 weeks (x---x) after the end of the diet.

The smallest concentration of ADP required to induce the second phase of aggregation, i.e. the ADP threshold, increased significantly after one week on the diet ( $p < 0.01$ ) and, although not significant after three weeks, remained increased for the duration of the diet, and three weeks thereafter.

## DISCUSSION

The results show that our previous observations<sup>5</sup> on prolongation of the bleeding time by comparatively small dietary changes are reproducible. The purpose of repeating the dietary experiment was to relate its effect on bleeding time to phospholipid composition and aggregability of platelets and to their production of thromboxane A<sub>2</sub> at the haemostatic site in vivo and in venous blood clotting in vitro.

The dietary fish increased  $\omega$ -3 and decreased  $\omega$ -6 fatty acids in the platelet membrane phospholipids within one week. The increased  $\omega$ -3 fatty acids were mainly C20:5 and C22:6 and the decreased  $\omega$ -6 fatty acids were mainly C18:2 (linoleic acid) and C20:4 (arachidonic acid). These changes persisted throughout the diet and reversed almost to control values three and eight weeks after its end.

These changes in the fatty acid composition of platelets were not matched by the changes that occurred in the haemostatic parameters. Not until six weeks on the diet was there an increase in the bleeding time (by 37%  $p < 0.01$ ); and this was still prolonged three weeks after the end of the diet, when the fatty acid composition of the platelets was back to normal. Furthermore, large differences in bleeding time prolongation in different individuals were associated with small differences in fatty acid composition (Table 1). Therefore the effects of the diet on the fatty acid composition of the platelets did not accompany its effect on the bleeding time.

The effects of the diet on the aggregability of platelets also failed to follow the changes in their fatty acid composition. Aggregation by collagen was diminished temporarily only after the first week on the diet as before<sup>5</sup>. Aggregation by ADP, on the other hand, was decreased throughout the

diet and for several weeks thereafter, i.e. even when the platelet fatty acid composition had reverted to normal, again as found before<sup>5</sup>. This makes it unlikely that the decreased platelet aggregability associated with fish diets is directly due to changes in the fatty acids of the platelets. Other investigations on fish oil supplements in the diet also indicate no consistent effect on platelet aggregation by collagen<sup>16, 17</sup> whereas platelet aggregation by ADP is decreased in human volunteers given salmon oil<sup>16</sup>, as well as in Eskimos<sup>18</sup> and in Japanese fisherman<sup>19</sup>.

Thus the effects of the fish diet on the fatty acid composition of the platelets did not accompany its effect on either in vivo bleeding time or on in vitro platelet aggregation. These results makes it unlikely that the increase in bleeding time depends directly on decreased platelet aggregability as measured in vitro or on changes in their  $\omega$ -3 fatty acids and they raise the possibility that as yet unidentified constituents of the fish diet are responsible for the haemostatic abnormalities. This possibility is supported also by observations that dietary intake of increasing amounts of EPA<sup>8, 17</sup>, did not increase the bleeding time further, and certainly not in a dose dependent manner.

It has been proposed that fish in the diet delays haemostasis by diminishing thromboxane  $A_2$  production by platelets<sup>3, 6, 8</sup>. We have already provided evidence against this<sup>5, 9, 10</sup>. To find out about this more directly, the Simplate technique for measuring bleeding times was adapted to permit quantification of thromboxane  $A_2$  in the blood emerging from the skin incisions. The fish diet caused no significant changes in this local appearance of thromboxane  $A_2$  in vivo, not even in those individuals whose bleeding times were increased most. This supports our conclusion that fish diets do not increase the bleeding time by diminishing the production of thromboxane  $A_2$  by the platelets involved in the haemostatic process. In striking contrast,

the production of thromboxane  $A_2$  in the same individuals venous blood clotting in vitro was significantly decreased within one week on the diet, throughout the diet and even for 18 weeks afterwards. Thus the time course of this decrease was independent of the effect on *in vivo* bleeding time supporting our earlier conclusion that determination of the capacity of clotting blood to form thromboxane  $A_2$  does not assist in evaluating the contribution of thromboxane  $A_2$  to the haemostatic situation<sup>15</sup>.

The decreased formation of thromboxane  $A_2$  in vitro agrees with similar decreases in human volunteers receiving either cod liver oil or corn oil<sup>20</sup>. Similarly also, in those on cod liver oil the thromboxane  $A_2$  formed in vitro had not returned to control values four months after the end of the diet.

One way to explain the discrepancy between the effect of the fish diet on thromboxane  $A_2$  formation in vitro and in vivo is through the assumption that in clotting venous blood all platelets are activated maximally and produce all the thromboxane  $A_2$  that can be formed from the arachidonic acid available for that conversion. That this is so is suggested by the similarity in thromboxane  $A_2$  formation in these experiments and in others<sup>20</sup> in which platelets were activated near maximally with collagen. Partial replacement of convertible arachidonic by other fatty acids from the fish diet would then reduce the amount of thromboxane  $A_2$  than can be produced.

This explanation can, however, only account for reduced thromboxane  $A_2$  formation while the fatty acid composition of platelet phospholipids is altered by the dietary fish, and not for the persistence in the reduction after the fatty acids have returned to normal. In vivo, on the other hand only a small proportion of platelets "at risk" are activated so that in bleeding time blood much less thromboxane  $A_2$  is formed and no difference appears between diet and control.



The persistence of the in vitro effects of the diet after its end could be explained by assuming that the diet alters some other, unknown factor or factors with biological time-constants sufficiently long for indirect expression through the parameters that were determined.

Our observations confirm that addition of fish to the diet causes primary haemostasis to be delayed, and that this delay does not depend on diminished local production of thromboxane  $A_2$ . The haemostatic abnormality cannot be accounted for directly by the increase of dietary  $\omega$ -3 fatty acids in the platelets. Haemostasis depends not only on platelets and their capacity to produce thromboxane  $A_2$  but also on the reactivity of the injured blood vessels and on the plasma coagulation mechanism. Therefore, dietary changes in these may be responsible for the prolongation of the bleeding time.

Our observations remain compatible with the proposition that dietary fish diminishes, by some mechanisms or other, the incidence of arterial thrombosis in human populations.

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